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WORLD REFERENCE CENTER FOR ARBOVIRUSES.(U)
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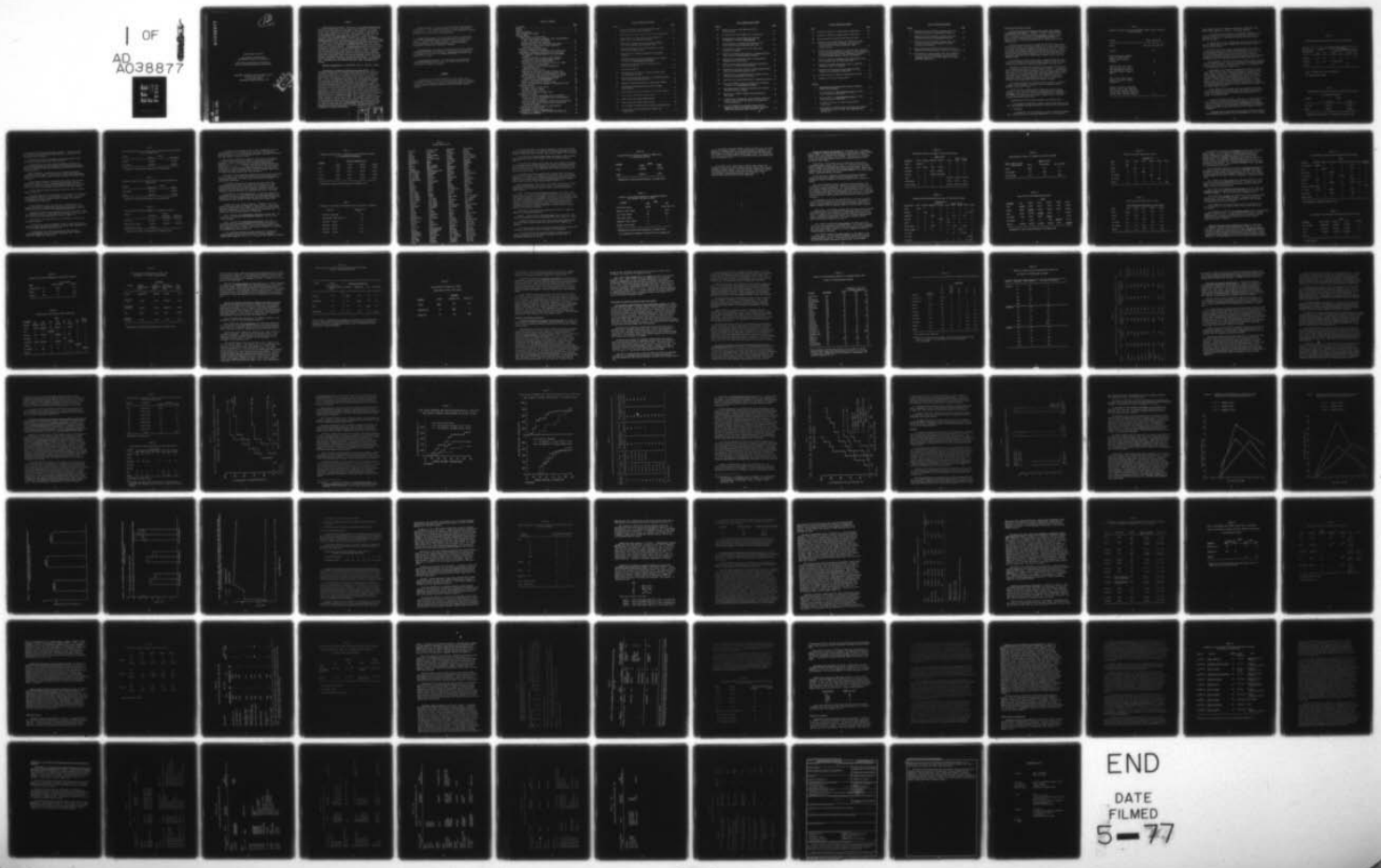

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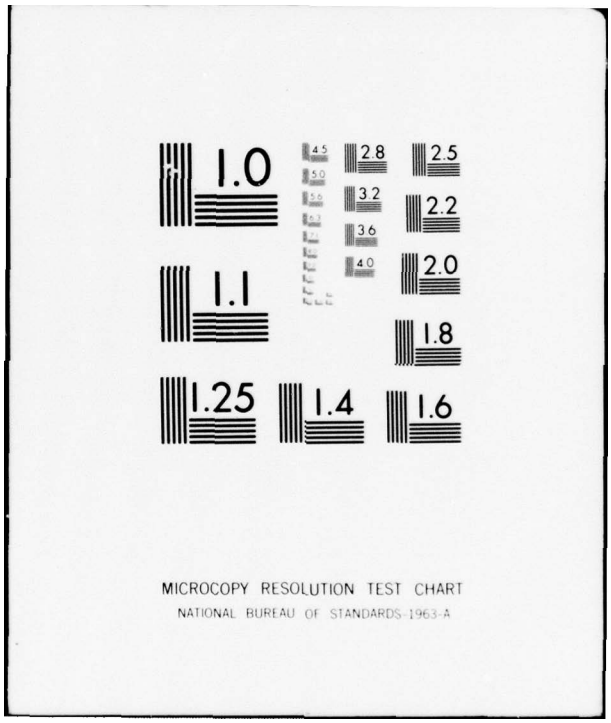
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Annual Report for 1976

Yale Arbovirus Research Unit
International Center for Arboviruses .

World Health Organization Collaborating
Center for Arbovirus Reference and Research .

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SUMMARY

Identification of viruses. Revisions of the Guama and Capim groups included identification of 2 new Guama group viruses and 3 new Capim group viruses from Brazil and Guatemala. Aruac, Kwatta, and Triniti viruses were recognized for the first time in Brazil. A new member of the California group, 2 new viruses in the phlebotomus fever group and 2 new viruses in the Simbu group were described. A possibly new arenavirus from the Amazon region of Brazil was studied. New rhabdoviruses from mosquitoes in Brazil, bats in Japan, mosquitoes in California, and Phlebotomus in Iran were characterized serologically. The Iran isolate is a member of the VSV serogroup. Ungrouped viruses from Brazil (X3), Malaysia, Panama, and Australia were tested with virtually all available arboviruses and found negative. A group B virus associated with Sao Paulo encephalitis differs by N test from other New World group B viruses and from all Old World Group B viruses so far tested. A Group B tick-borne virus from Australia is closely related to Tyuleniy virus. A Hughes group virus was recognized for the first time in South Africa and other tick-borne viruses were identified from new geographic areas: Dhori from Portugal, Abu Hammad from Iran, and Bhanja from Bulgaria. A Jos-related virus from Kenya and a Nyamanini-related virus from Japan were characterized. Apparently new tick viruses from USSR, Kenya, and Uganda were studied.

Mosquito transmission was accomplished with the orbivirus, Orungo.

Development of arbovirus techniques and animal models. Salt-dependent hemagglutination of Bunyaviridae antigens has been found for 9 different serogroups. The effect is additive to that of sonication. Glutaraldehyde-fixed goose cells which can be stored up to a year, agglutinate a wide variety of arboviruses but some HI sensitivity is lost. Titers of arenaviruses are higher with low-multiplicity passage than with high multiplicity in VERO cells and the soluble CF antigen was separated from infectivity by gradient centrifugation. Indirect immunofluorescence with arenaviruses has proved highly sensitive. The degree of cross-reactions in some cases presents diagnostic difficulties but offers the use of relatively non-hazardous viruses for diagnosing Lassa fever. A Junin virus-mouse model for tolerant arenavirus infection demonstrated an active effect of Junin virus on the spleen lymphocyte. Mouse sarcoma cells infected with Congo-CHF virus were used as a sensitive diagnostic system in immunofluorescence tests. Varma's Ixodid tick tissue culture was established at YARU. The sarcoma-180 method produced larger volumes of ascitic fluid in mice than Freund's adjuvant but did not yield greater absolute amounts of antibody. Fluorescent focus and its inhibition was developed in Aedes albopictus and in vertebrate cells for assay of arboviruses and their antibody. The CER and mouse neuroblastoma cells have proved superior to other vertebrate lines for propagation of several arboviruses.

Serologic surveys. Serologic study of 400 sera of missionaries from West Africa showed continued activity of Lassa, Congo, Bwamba, chikungunya and dengue viruses and a survey of sera from Ghana indicated infections with alpha, flavi, and bunyaviruses.

Diagnosis of disease. The HI test using goose and trypsinized human O cells diagnosed Crimean hemorrhagic fever in patients from Pakistan. Attempts to diagnose outbreaks or suspected cases of Korean hemorrhagic fever, arthrogryposis, Alzheimer's disease, Legionnaire's disease, and dengue were negative.

Lyme arthritis. Serologic and virologic tests of Lyme arthritis (Connecticut) patients were negative. The distribution of deer and the abundance of the deer tick, Ixodes scapularis, correlated with distribution of cases.

Distribution of reagents. The Yale Arbovirus Research Unit distributed reference sera, viruses, cell lines, and under some circumstances, diagnostic antigens.

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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Identification of virus strains

Viruses from Australia, Portugal, USSR, Kenya, Iran, Uganda, Czechoslovakia, and Japan. (J. Casals). Group B. Strain CSIRO-4, submited by Dr. R.L. Doherty, Brisbane, Australia (studied at YARU with J. Brandsma), was isolated from Ornithodoros capensis from a gannet's nest on Sumarez Reef off the central Queensland coast in September 1974; it had been determined by Dr. Doherty that it was a Group B virus.

Screening against 50 group B viruses was done by CF test using an immune mouse ascitic fluid against CSIRO-4, in dilutions beginning at 1:8, against the Group B antigens in dilutions 1:8 and 1:32; the results of several tests have been combined and are shown in Table 1. These tests showed that CSIRO-4 ascitic fluid reacted with the highest cross-reacting titer with Tyuleniy antigen, 1:64, and with lower but still reasonably high titers with, among others, several of the tick-borne complex viruses.

As reported in the 1975 annual report, complete reciprocal grid titrations of CSIRO-4 and Tyuleniy viruses showed that they are indeed closely related viruses, although distinguishable from each other.

Comparison of CSIRO-4 and Tyuleniy by intracerebral neutralization test in newborn mice, gave the results shown in Table 2. It appeared that a high titered Tyuleniy antiserum with a homologous neutralization index (NI) of 6.0 or higher gave good protection against CSIRO-4 virus, even though at a somewhat lower level, with heterologous NI of 4.8. The immune ascitic fluid for CSIRO-4, while giving a satisfactory protection against the homologous virus, NI of 3.5, failed in two attempts to give significant protection against Tyuleniy.

It can be concluded that, based on the above results, CSIRO-4 is an agent closely related to Tyuleniy virus but separable from it, possibly a new Group B virus.

Strain Poti 461, submitted by Dr. Armindo R. Filipe, Lisbon, Portugal. This is one of 5 similar strains isolated from Hyalomma marginatum ticks taken off cattle in the slaughterhouse; according to Dr. Filipe, all precautions were taken to insure that no laboratory cross-contaminations would occur.

Identification of this strain included the following steps:

1. A homologous CF system was prepared; mouse immune ascitic fluid and a sucrose-acetone antigen had titers of 1:256 and 1:64, respectively

2. Screenings

a. Antigen Poti 461 in dilutions 1:4 and 1:8, tested with immune sera or ascitic fluids, in dilutions beginning at 1:8, for groups B,

Table 1

Complement fixation test between CSIRO-4 immune ascitic fluid and group B antigens

Antigen	Titer, reciprocal
CSIRO-4	128, 256, 256, 256, 256
Tyuleniy	64
RSSE, Louping ill, Negishi, Uganda S, yellow fever, Karshi, Usutu, Sokuluk, Koutango	32
Edgehill, MVE, Zika, Alfuy, Banzi, Ilheus, Apoi, SLE, OHF, Wesselsbron, West Nile, Spondweni, Saboya, dengue 2, CETB	16
KFD, Kunjin, Kadam, Langat, dengue 4, Israel TM, Ntaya, Entebbe bat	8
Dengue 3, Kokobera, Bussuquara dengue 1, Modoc, MML, Pnom Pehn bat, Tembusu, Jutiapa, Stratford, US bat, Royal Farm, JE, Dakar bat, Cowbone Ridge, Powassan, Bouboui	<8

Guama, Simbu, California, Vesicular stomatitis, phlebotomus fever, Capim and NIH numbers 1, 3 and 12. All results negative.

b. Ascitic fluid for Poti 461, in dilutions beginning at 1:8, against antigens for 53 tick-borne viruses outside of group B, in dilutions 1:4 and 1:16. Only positive results were with antigens Poti 461 and Dhori.

3. Identification by CF. Complete box titrations with Dhori and Poti 461 immune sera or ascitic fluids and their antigens, gave the result shown in Table 3.

Dhori and Poti 461 are nearly indistinguishable by complement-fixation test; pending the outcome of neutralization tests, Poti 461 is provisionally considered to be a strain of Dhori. This is an interesting observation; since Dhori virus was not, presumably, stocked in the Lisbon Laboratory, this is considered a valid isolation. Heretofore, Dhori had only been isolated in India, Egypt and USSR.

Strain #503095. Submitted by Dr. Robert B. Tesh, Pacific Research Section, NIAID, Honolulu. The strain was isolated from Argas hermanni ticks (adults and nymphs) collected off the walls of a bird house in a village--Dormian--in Iran, August 1975.

A complement-fixing antigen was prepared from infected newborn mouse brain tissue by the sucrose-acetone method; and also a mouse immune serum, 2 injections. Screening against 54 non-Group B tick-borne antigens revealed that the immune serum for strain #503095 reacted only with the following antigens: Abu Hammad, Abu Mina and Pretoria. These viruses are a complex within the DGK antigenic group. Since the cross-reaction between #503095 serum and antigen Abu Hammad was greater than with the other two antigens, a complete CF cross-titration was carried out between #503095 and Abu Hammad, with the result shown in Table 4.

Strain #503095 and Abu Hammad virus are identical by CF test; while final identification should await the result of neutralization tests, the isolate from Iran is provisionally considered to be a strain of Abu Hammad virus.

Strains CZ-335/336 and CZ-326. These isolates were submitted by Dr. V. Bardos, Institute of Parasitology, Prague, Czechoslovakia. These strains were isolated from ticks, Haemaphysalis S. punctata, collected in Bulgaria; as they appeared to be identical by complement-fixation test, most of the identification work was done with strain CZ-335/336.

A sucrose-acetone antigen and a mouse immune serum were prepared with CZ-335/336; the serum in dilutions beginning at 1:8 was screened

Table 2

Neutralization tests in mice by ic route of inoculation

Serum or ascitic fluid, all diluted 1:3	Virus, in dilution					
	CSIRO-4		Tyuleniy			
			Test #1		Test #2	
	LD_{50}	NI	LD_{50}	NI	LD_{50}	NI
CSIRO-4	4.0	3.5	6.0	1.5	6.2	0.8
Tyuleniy	2.7	4.8	1.5,less	6.0+		
Control	7.5		7.5		7.0	

LD_{50}, ic lethal dose; log of reciprocal

NI, neutralization index

Table 3

Identification of Poti 461 virus as a strain of Dhori virus, by complement fixation

Antigen	Serum	
	Poti 461	Dhori
Poti 461	128/128	256/64
Dhori	128/512	512/512

Reciprocal of serum titer / reciprocal of antigen titer.

by CF against 52 non-group B tick-borne antigens. The only antigen with which the immune CZ-335/336 serum reacted, in addition to the homologous one, was Bhanja.

A reciprocal, complete box-titration between CZ-335/336 strain and Bhanja virus gave the result shown in Table 5.

It appears that by CF test, strain CZ-335/336 and Bhanja are identical; unless they were substantially different by neutralization test, it is concluded that the two isolates from Bulgaria are strains of Bhanja virus.

Strain Jap-Cap-44. Submitted by Dr. M. Takahashi, National Institute of Health, Tokyo. The strain was isolated from Ornithodoros capensis ticks, collected in gulls' nests, in Japan (38°N, 141E), Iwate Prefecture.

A mouse immune serum and a sucrose-acetone antigen were prepared both with CF titers of 1:128. Screening of the serum by CF test, against 55 non-group B tick-borne virus antigens showed that the serum reacted only with its homologous antigen and with Nyamanini antigen.

A set of complete box-titrations was carried out with the results shown in Table 6.

The results show that strain Jap-Cap-44 is related to but distinct from Nyamanini; whether or not Jap-Cap-44 is a new virus, or a strain of Nyamanini, or perhaps an agent similar to Midway virus remains to be determined by more work.

Strains USSR Cg 4, Cg 8, Cg 12 and Cg 13, submitted by Dr. A. Butenko, Institute of Poliomyelitis and Virus Encephalitides, Moscow. The strains had been isolated from Clethrionomys glareolus in 1974-75.

Complement-fixing antigens and immune mouse sera and ascitic fluids were prepared with all; a cross-fixation test showed that by this test the 4 strains were indistinguishable as shown in Table 7.

Additional CF tests were carried out in attempts to identify the strains as follows:

1. Strain Cg 13, serum and antigen, failed to cross react with ectromelia, hepatoencephalitis (Reovirus 3), herpes, NDV, paramyxoviruses 1, 2, 3 and SV5, and mouse hepatitis virus.

2. Convalescent sera from six patients who had survived a clinically diagnosed attack of Korean hemorrhagic fever, failed to react with antigen for strain Cg 4.

Table 4

Complement fixation test; cross-reaction between Iran strain #503095 and Abu Hammad virus

Antigen	Serum	
	#503095	Abu Hammad
#503095	256/512	256/512
Abu Hammad	256/256	256/512

Reciprocal of serum titer/reciprocal of antigen titer

Table 5

Complement fixation test; identification of strain CZ-335/336 as Bhanja virus

Antigen	Serum	
	CZ-335/336	Bhanja
CZ-335/336	512/256	128/256
Bhanja	256/512	128/256

Reciprocal of serum titer/reciprocal of antigen titer

Table 6

Identification of strain Jap-Cap-44 by complement fixation

Antigen	Serum		
	Jap-Cap-44	Nyamanini (Ar 1304)	Nyamanini (tick 39)
Jap-Cap-44	128/128	64/128	64/128
Nyamanini (Ar 1304)	32/256	256/1024	256/1024
Nyamanini (tick 39)	32/64+	-	

Reciprocal of serum titer/reciprocal of antigen titer

3. An immune ascitic fluid for Cg 13, with a homologous titer of 1:32, failed to react, at dilution 1:2, with any of 216 antigens, including representatives of all arbovirus groups, ungrouped arboviruses and other agents, as listed in Table 9 .

The size was estimated by sequential filtration of a suspension of infected newborn mouse brain tissue through graded Milipore(R) membranes. A 10^{-2} suspension of Cg 8 strain was centrifuged at 4000 rpm for 30 minutes and passed in succession through filters with average pore diameters (APD) of 450, 220, 100 and 50 nm; the filtrates as well as the original uncentrifuged suspension and the suspensions centrifuged at 4000 X 30 minutes, were titrated in newborn mice by the ic route; the titers obtained are given in Table 8 .

The result of this experiment indicates that the infectious particle of Cg 8 is large, for a virus; according to our previous experience with numerous viruses, the size in this case should be estimated to be between 150 and 300 nm.

A preliminary observation by electron microscopy done with infected mouse brain tissue (the strain did not replicate in VERO cells) showed only one or two particles that resembled a virion, round-oval in shape about 110 nm in diameter; additional studies are planned in order to determine the morphology and size of this agent.

Susceptibility to sodium deoxycholate. A test with strain Cy 8 showed that the strain is inactivated by 0.1% sodium deoxycholate; the $LD_{50}/0.02$ of a virus suspension was less than 10^{-2} where incubated in the presence of SDC, and $10^{-5.0}$ when incubated with diluent only.

Strains T-39, F-83, T-100 and Makindu. Submitted by Dr. F.G. Davies, Veterinary Research Laboratory, Kabete, Kenya. These strains were sent to YARU with a fair amount of information concerning their antigenic relationships, which fact greatly facilitated our task. The work so far done with these agents is summarized.

T-39. Isolated from Rhipicephalus pulchellus, January 1974. No virus could be recovered from the sample submitted, not even after 2 blind passages.

T-83. Isolated from Rhipicephalus pulchellus, March 1974. A sucrose-acetone antigen was prepared, which failed to agglutinate goose cells but had a CF titer of 1:64; a mouse immune ascitic fluid had a homologous titer of 1:128. With these two reagents the following CF screening tests were carried out.

1. No cross-reactions were observed between T-83 antigen and grouping immune ascitic fluids for groups B, Palyam, polyvalent Johnson Atoll, Kemerovo, and #1, 3, 7, 10 and 12 (NIH reagents catalogue).

Table 7

Complement-fixation test. Similarity of four strains isolated from Clethrionomys glareolus

Antigen	Serum or ascitic fluid			
	Cg 4	Cg 8	Cg 12	Cg 13
Cg 4	8/64*	16/64	32+/64+	32/64
Cg 8	8/16	16/16	32/16	16/32
Cg 12	8/32	16/32	32+/16	32/32
Cg 13	8/32	16/32	32/32	32/64

*Reciprocal of serum titer/reciprocal of antigen titer

Table 8

Estimation of particle size of strain Cg 8 by Milipore filtration

Material	$LD_{50}/0.02$ ml 10^{-}
Original suspension	5.6
Supernatant, 4000 rpm x 30	5.5
Filtrate 450 nm	4.9
Filtrate 220 nm	2.2
Filtrate 100 nm	<1.5
Filtrate 50 nm	<1.5

Table 9

Viruses Tested by CF

Abu Mina
Acado
Acara
Akabane
Amapari
Anhanga
Anopheles A
Anopheles B
Apeu
Arkonam
Aruac
Arumowat
Aus MRM 4059
Aus Ar 8659
Aus C 12048
Aus CH 9935
Aus MK 6357
Aus MRM 10434
Bahig
Bakau
Bandia
Bangoran
Bangui
Bauline
BeAn 67949
BeAn 84381
BeAn 100049
BeAn 141106
BeAn 174214
BeAn 177325
Bertioga
Bhanja
Bluetongue
Bobia
Bocas
Boteke
Botembe
Bujaru
Bushbush
Buttonwillow
Bwamba
California
Candiru
Capim
FG Ar 564
Chaco
Chagres
Chandipura
Changuinola
Charleville
Chenuda
CHF-Congo
CoAr 1279
Cocal
Colorado tick
Corriparta
Cotia
Cowbone Ridge
Dengue 2
Dera Ghazi Khan
Dhori
Dugbe
EEE
EgAn 1398-61
EgAn 1825-61
EHD-NJ
Eretmapodites 147
Eubenangee
Flanders
Gamboa
Germiston
Gossas
Grand Arbaud
Guajara
Guama
Guaroa
Hazara
Herpes
Hilo
Huacho
Hughes
I 6235
I 61-2629
IbAn 8341
IbAn 20433
IbAn 28946
IbAn 38918
Ichampadi
Ieri
Ilesha
Irituia
Itaporanga
Itaqui
Joinjakaka
Jos
Junin
Jurona
Kaeng Khoi
Kairi
Kammavanpettai
Kannamangalam
Karimabad
Kemerovo
Kern Canyon
Keterah
Klamath
Koongol
Kotonkan
Kowanyama
Kwatta
Lagos bat
La Joya
Lanjan
Lebombo
Le Dantec
Lone Star
Lukuni
LCM
Manzanilla
Mapputta
Marco
Matariya
Matucare
Melao
Minititlan
Minnal
Mirim
MML
Modoc
Mokola
Mossuril
Mt Elgon bat
Nariva
Navarro
NDV
Nkolbisson
Nola
Nyamanini
Nyando
Okola
Olifantsvlei
Oropouche
Oyo
Pacora
Pacui
Palyam
Pata
Patois
Pichinde
Powassan
Punta Toro
Qalyub
Quaranfil
Rabies
Reovirus
Rio Bravo
Salehabad
SF - Naples
SF - Sicilian
Sathuperi
Sawgrass
Sembalam
Silverwater
Simbu
Soldado
Sororoca
Sud An 754-61
Sud Ar 1169-64
Tacaiuma
Tamiami
Tataguine
Tembe
Tensaw
Tete
Thimiri
Thogoto
Thottapalayam
Timbo
Triniti
Trivittatus
Tsuruse
Turlock
Ug MP 359
Umbre
Upolu
Utinga
Uukuniemi
Vaccinia
VEE
VS - New Jersey
VS - Indiana
Wad Medani
Wanowrie
WEE
Witwatersrand
Wongal
Wyeomyia
Yaba - 1
Yata
Yellow fever
Yogue
Caraparu
Makindu
Ug Sq 37317/9
EgArT 904
Indo 1039
USSR Cq 13
Ken T 83
Tettnang
EgArT 1147
Mal P 350
Eg ArT 427
Han 70-3-28
P 72-4R
BFN 3187
Oita 296

2. No cross-reactions were detected between an immune ascitic fluid for T-83 (homologous titer of 1:128) and the following antigens: Kemerovo, Chenuda, Huacho, Mono Lake, Bauline, Great Island, Hughes, Dhori and Zirqa.

3. No cross-reactions were noted between an immune ascitic fluid for T-83 (homologous titer 1:128) and any of the 216 antigens tested in Table 9.

4. The same T-83 immune ascitic fluid was tested against 54 antigens for tick-borne viruses outside group B; the ascitic fluid gave negative reactions with all except the homologous antigen and the antigen for Jos virus; the latter was at a very low level with a titer of 1:8 for the AF and 1:4 for the antigen.

5. In view of the trace cross-reaction observed above (4) between T-83 AF and Jos antigen, a complete set of reciprocal box titrations was carried out. In these were included antigens for Jos derived from liver tissue as well as from brain, the former being known to have a higher titer than the latter; the results are shown in Table 11.

The crossing between T-83 and Jos is so small, even though it seems technically without flaw, that at this time it is considered only a possibility; the observation requires further study using reagents of higher potency.

T-100. Isolated from Rhipicephalus pulchellus, April 1974. The strain was sent to YARU with the information that it cross-reacted with Kadam virus; therefore the request was to confirm this relationship. This was easily accomplished; a screening by CF was done using an immune serum for T-100 in dilutions beginning at 1:8 and antigens for 52 group B viruses in dilutions 1:8 and 1:16. The highest cross-reacting titer given by the serum was with Kadam antigen. A complete set of box-titrations with the two viruses, Kadam and T-100 was carried out next, which is illustrated in Table 10.

The result of this cross-test confirms that T-100 is a strain of Kadam, by complement-fixation test.

Makindu. Isolated from a mixed Culicoides pool, April 1974. The strain was submitted with the information that it was resistant to ether.

A complement-fixing antigen and a mouse immune serum were prepared with this virus; screening tests were done with these reagents, as follows:

1. No cross-reaction was noted between Makindu serum and antigen for: D'Aguilar, Kasba, Palyam, Acado, Corriparta, and Eubenangee.

2. No cross-reaction was observed between an immune serum for Makindu (homologous titer of 1:64) and antigens for fifty-five tick-borne viruses not of Group B.

Table 10

Identification of T-100 as a strain of Kadam virus by complement-fixation

Antigen	Serum	
	T-100	Kadam
T-100	128/128*	32/128
Kadam	128/182	32/64

*Reciprocal of serum titer/reciprocal of antigen titer

Table 11

Possible cross-reaction detected by complement-fixation test between T-83 and Jos virus

Antigen	Sera	
	T-83	Jos
T-83, brain tissue	32/32*	Trace only, 4/2
Thogoto, liver tissue	0	64/32
Jos, liver tissue	4/8	32/4
Jos, brain tissue	0	0
Caraparu, liver tissue	0	0
Normal, brain tissue	0	

*Reciprocal of serum titer/reciprocal of antigen titer;

0, no fixation at dilution of serum 1:4 and of antigen 1:2.

3. In another screening, Makindu serum at dilution 1/4, was tested against 216 antigens (Table 9); the only antigen, in addition to the homologous, with which the serum reacted was one prepared from strain Eg An 1398-61, isolated in Egypt in 1961 from a bird. The observation, it is felt, needs confirmation and elaboration before much significance can be attributed to it.

Ug Sg 37317 virus isolated from a febrile human patient was referred to YARU by Dr. P.A.K. Addy of the East African Virus Research Institute, Entebbe, Uganda. The virus has been tested with a battery of grouping ascitic fluids and the mouse ascitic fluid with 216 viral antigens by complement-fixation tests (Table 9). All reactions were negative except for the homologous.

Viruses from Brazil and Guatemala (A. Travassos, and J. Travassos with R. Shope). Fifteen viruses referred by the Instituto Evandro Chagas, Belem, Brazil, and 3 viruses from Guatemala submitted by Dr. William Scherer of Cornell University were studied. The study led to revisions of the Capim and Guama groups as well as to characterization of new viruses in other groups.

Capim Group. Be An 153564 and Be An 84381 from sentinel mice exposed near Belem, Brazil, and 71 U 344, 71 U 253, and 71 U 350 from sentinel hamsters in Guatemala were known to be Capim group viruses by virtue of their reaction in CF with a Capim grouping fluid. These were compared by CF, HI, and NT with the described members of the group, Capim, Guajara, Bushbush, Moriche, and Acara.

By CF there were 2 distinct complexes--Capim-Guajara-Bushbush-Moriche and Acara-Be An 84381 and Be An 153564 (Table 12). The Guatemalan isolates belonged to the Acara complex by CF (not shown in Table).

By HI the Trinidad and Brazil isolates are quite distinct. The 71 U 253 and 71 U 350 isolates are closely related and surprisingly, cross-react strongly with Guajara. The other Guatemalan virus, 71 U 344 is a subtype of Be An 84381 (Table 13). Neutralization tests at Cornell had previously shown the close relationship of 71 U 253 to 71 U 350, and of 71 U 344 to Be An 84381. Neutralization tests with Acara, Be An 84381, and Be An 153564 at YARU confirmed their distinctness from each other (Table 14).

The divergence of CF and HI relationships of 71 U 350 (related to Acara by CF and to Guajara by HI) may be a function of reassortment in nature of genome segments, with the soluble CF antigen coded for by a segment different from the segment coding for virion surface antigens.

Guama group. Be An 109303 isolated from Caluromys and Be An 116382 from sentinel mice, were recognized as Guama group viruses in Belem, Brazil but appeared to be distinct from other Brazilian members of the group. A revision of the group using CF, HI, and mouse neutralization tests confirmed the distinctness of the newly recognized members. Results are shown in Tables 15, 16, and 17.

Be Ar 202527 isolated from Culex portesi was shown by CF and N tests to be indistinguishable from Aruac virus. Be An 235467 isolated from blood of an agouti was shown by CF and N tests to be indistinguishable from Trinitì virus. This is the first recognition of these 2 viruses outside of Trinidad.

Be Ar 103645 isolated from Aedes fulvus is a new member of the California group. A CF test with 1 injection hamster sera gave the results shown in Table 18. In another CF test the virus was shown to differ from Inkoo, San Angelo, Snowshoe hare, and La Crosse.

Table 12

Complement fixation reactions of Capim group viruses

	Sera (mice)						
Antigens	Capim	Guajara	Bushbush	Moriche	Acara	BE AN 84381	BE AN 153564
Capim	64/≥256	0	0	0	0	0	0
Guajara	8/16	32/16	8/16	32/16	0	0	0
Bushbush	0	0	32/16	≥256/16	0	0	0
Moriche	0	0	32/≥256	≥256/≥256	0	0	0
Acara	0	0	0	0	16/≥256	16/≥256	16/≥256
BE AN 84381	0	0	0	0	8/64	32/64	8/64
BE AN 153564	0	0	0	0	16/≥256	32/≥256	32/≥256

Table 13

Hemagglutination-inhibition reactions of Capim group viruses

	Antigens (4u.)								
Sera (mice)	Capim	Guajara	Bushbush	Moriche	Acara	BE AN 84381	BE AN 153564	71U344	71U350
Capim	1:80	0	0	0	0	0	0	0	0
Guajara	0	1:80	0	0	0	0	0	0	≥1:160
Bushbush	0	0	1:80	0	0	1:20	0	1:10	0
Moriche	0	0	0	1:160	1:40	0	0	0	0
Acara	0	0	0	0	1:40	0	0	0	0
BE AN 84381	0	0	1:20	0	0	1:80	0	1:40	0
BE AN 153564	0	0	0	0	0	0	1:40	0	0
71 U 344					0	40	0	1:320	0
71 U 253									≥1:320
71 U 350					0	0	0	0	≥1:320

Table 14

Neutralization tests of 3 viruses in the Acara complex

Virus (infected baby mouse serum)	Sera (log NI)		
	Acara	BE AN 84381	BE AN 153564
Acara	>6.2	0.1	0.1
BE AN 84381	1.2	>5.1	0.1
BE AN 153564	1.2	0.1	>5.1

Table 15

Cross CF test with Guama group viruses

Antigens	Sera					
	Catu	Guama	Moju	109303	116382	Bimiti
Catu	64/64*	64/64	64/16	64/16	8/16	128/64
Guama	32/64	64/256	64/64	64/64	8/64	128/64
Moju	16/64	64/256	64/256	64/64	0	64/256
BE 109303	8/64	32/64	32/64	128/64	0	32/64
BE 116382	0	4/64	0	8/64	256/256	16/64
Bimiti	32/256	64/256	64/256	64/256	16/64	128/256

*Reciprocal of serum titer/reciprocal of antigen titer.

Table 16

Cross HI test with Guama group viruses

	Antigens (4u.)					
Sera	Catú	Guamá	Mojú	109303	116382	Bimiti
Catú	160	0	0	0	0	0
Guamá	0	80	20	40	0	0
Mojú	0	20	160	0	0	0
BE 109303	0	20	0	160	0	0
BE 116382	0	0	0	0	160	0
Bimiti	0	0	0	0	0	160

Table 17

Cross N test with Guama group viruses

	Virus (infective serum used as virus source)				
Sera	Catú	Guamá	Mojú	109303	Bimiti
Catú	≥5.0	≤1.2	≤0.9	≤0.5	≤1.1
Guamá	≤1.1	4.4	2.1	2.5	≤1.1
Mojú	≤1.1	≤1.2	≥3.9	1.5	≤1.1
BE 109303	≤1.1	3.4	1.9	5.5	≤1.1
BE 116382	≤1.1	≤1.2	≤0.9	≤0.5	≤1.1
Bimiti	≤1.1	≤1.2	≤0.9	≤0.5	5.6

Be An 213452 was isolated from Didelphis captured along the Trans Amazon highway. It was found at the Belem Virus Laboratory to be closely related by CF to Candiru of the phlebotomus fever group. CF tests at YARU were negative with Naples, Sicilian, Arumowot, Chagres, Punta Toro, Karimabad, Anhanga, Bujaru, Icoaraci, and Sud An 754-61 viruses. Cross-reactions observed with Candiru and Nique viruses are shown in Table 19. In a neutralization test Be An 213452 and Candiru were distinct. Be An 213452 is presumed to be a new member of the phlebotomus fever group.

Be An 157575 was isolated from plasma of Pyriglena leucoptera and was shown in Belem to cross-react by CF with the rhabdovirus, Kwatta. Reciprocal CF tests showed 4-fold differences each way. The results of mouse neutralization tests however indicate the viruses are closely related (Table 20), and Be An 157575 is considered a sub-type of Kwatta virus.

Be Ar 185559 from Culex portesi was indistinguishable by CF test from a virus isolated in Colombia, Co Ar 1279. CF tests with a large number of viruses showed minor cross-reactions with Hart Park and Kamese viruses, both rhabdoviruses.

Be Ar 177325 from Lutzomyia flaviscutellata and Be An 174214 from Philander opossum were negative by CF with grouping sera and with 192 individual antigens. They are presumed new, ungrouped viruses.

Be An 238758 from Oryzomys was negative by CF with grouping fluids and with most of 192 individual antigens. A puzzling low-titer CF reaction with 2 strains of Congo-Crimean hemorrhagic fever antigens is unexplained (but will not go away).

Be An 228950 from a frog was viable, but had a maximum titer of 1.8 log LD_{50}/.02 ml. This agent is still being studied and may not be a virus.

Be An 293022 was isolated from Oryzomys oecomys along the Trans Amazon highway in November, 1975. It reacted in Belem by CF with a Tacaribe grouping fluid. A CF test at YARU showed it to be distinct from the arenaviruses tested (Parana and Latino viruses not yet tested). Results are shown in Table 21.

Rhabdoviruses from Iran, California, and Japan (R. Shope with J. Brandsma). Isfahan virus was isolated by R. Tesh and S. Saidi from pools of sandflies which were predominantly Phlebotomus papatasi and shown in Hawaii to be neutralized by a VSV grouping ascitic fluid. CF tests at YARU confirmed the relationship to the VSV group. Cross-reactions were seen, however, only with concentrated antigen dilutions (Table 22). By neutralization test in mice using undiluted hyperimmune

Table 18

BE AR 103645 CF reactions with California group viruses

	Sera						
Antigens	AR103645	Melao	California	Trivittatus	Keystone	Tahyna	Jamestown Canyon
BE AR 103645	64/256*	0	0	0	8/64	0	0
Melao	0	16/256	0	0	8/64	0	0
California	0	0	32/256	0	0	0	0
Trivittatus	0	0	8/4	512/16	8/4	0	0
Keystone	8/64	0	0	0	32/64	0	0
Tahyna	0	0	128/16	8/4	16/16	32/16	0
Jamestown Canyon	16/64	0	0	0	0	0	16/64
La Crosse	16/64	0	32/16	0	16/4	0	0
San Angelo	0	0	8/64	0	8/64	0	0
Normal brain	0	0	0	0	0	0	0

*serum/antigen; 1 injection hamster sera.

Table 19

CF reactions of BE AN 213452 with Candirú and Nique viruses

	Sera			
Antigens	BE AN 213452	Candiru	Nique	Control
BE AN 213452	≥256/≥256*	64/≥256	64/≥256	0
Candirú	≥256/≥256	64/≥256	64/≥256	0
Nique	16/≥256	8/≥256	≥256/≥256	0
Control	0	0	0	

*serum/antigen

Table 20

Results of NT test with Kwatta and BE AN 157575 viruses

Sera		Virus (LNI) BE AN 157575	Kwatta
BE AN 157575	1:8	≥2.8	≥4.7
Kwatta	1:8	2.1	3.0
Control	1:8	0	<0.9

Table 21

CF reactions of BE AN 293022 with arenaviruses

Antigens	Sera AMA	JUN	LCM	MAC	PIC	TCR	TAM	BE AN 293022
Amapari	64/≥40	≥64/≥40	0	32/≥40	0	16/≥40	0	0
Junin	32/≥40	≥64/≥40	8/4	≥64/≥40	0	16/≥40	0	0
LCM	0	0	≥64/≥40	0	0	0	0	0
Machupo	8/4	≥64/≥40	0	≥64/≥40	0	16/4	0	0
Pichinde	0	0	0	0	8/4	0	0	0
Tacaribe	8/≥40	≥64/≥40	0	≥64/≥40	0	64/≥40	0	0
Tamiami	0	0	0	0	0	0	≥64/≥40	0
BE 293022	0	8/4	0	0	0	0	0	16/≥16
Normal	0	0	0		0	0	0	0

Table 22

CF reactions of Isfahan Virus with Piry and Chandipura of the genus Vesiculovirus

Antigen	Antibody			
	Isfahan mouse ascitic fluid	Isfahan guinea pig serum	Chandipura mouse ascitic fluid	Piry mouse serum
Isfahan	1024/1024*	256/256	16/16	8/4
Chandipura I 653514	64/256	32/16	256/1024	16/64
Chandipura IbAn 9978	32/256	32/16	256/1024	16/16
Piry	64/16	8/4	16/4	512/64
Flanders (Control)	<4/<4	<4/<4	<4/<4	<4/<4

*Reciprocal of antibody titer/reciprocal of antigen titer.

ascitic fluids or sera, again cross-reaction was demonstrated with several vesiculoviruses (Table 23). A HA antigen was produced in CER cells grown in inhibitor-free medium. The antigen titered 1:256 at pH 6.2, 4°C and was inhibited specifically by homologous antibody. Isfahan virus is a new member of the VSV group.

BFN 3187 from Culex tarsalis was submitted by Dr. J. Hardy, University of California and Oita-296 from a bat was submitted by Dr. A. Oya of the NIH, Tokyo, Japan. Both viruses were known to be rhabdoviruses by EM. Ascitic fluids of BFN 3187 and Oita 286 were tested by CF with 216 antigens listed in Table 9 . The homologous reactions were positive. A positive CF reaction of BFN 3187 with EgAr T 1147 is believed to be non-specific. Otherwise all tests were negative and the 2 rhabdoviruses are presumably new.

Viruses from Panama, South Africa, Malaysia, Australia and Brazil (R. Shope and W. Downs with S. Brown, K. Palmer and S. Ofori-Kwakye). Bradypus 121 and Bayano 517 viruses from a sloth and from Culicoides respectively were sent by Dr. C. Seymour of the Gorgas Laboratory. Using reagents prepared in Panama, the CF reaction with Simbu grouping fluid was confirmed. By CF test the 2 viruses were indistinguishable from Utinga virus. HI tests (Table 24) showed that the viruses are distinguishable from each other and from Utinga virus.

Sera of another virus submitted by Dr. Seymour, Bradypus 4, was negative at 1:8 by CF with 216 antigens (Table 9).

Sa Ar 15908 virus from Ornithodoros bird ticks was submitted by Dr. B. McIntosh, of the South African Institute of Medical Research. A serum sent by Dr. McIntosh titered 1:16 by CF with Soldado antigen. This is the first record of a Hughes group virus from South Africa.

P72-4R virus from a monkey was referred by Dr. Albert Rudnick of the Hooper Foundation. This agent was inactivated by DCA. It was negative by CF with 14 grouping fluids and did not react with 216 individual antigens (Table 9) except for the homologous. It is presumably a new, ungrouped virus.

Dak An B496 (Gordil) virus was referred by Dr. Y. Robin of the Institut Pasteur, Dakar. It was known to be in the phlebotomus fever group and was isolated from the same animal as St. Floris virus. An B496 was negative by CF with 30 grouping fluids. HA antigen titered 1:640 at pH 6.3. In an HI test, the phlebotomus fever grouping fluid inhibited 1:10, other grouping fluids were negative. By CF the following phlebotomus fever group antigens failed to react with Dak An B496 antibody: Anhanga, Arumowot, Be An 100049, Bujaru, Candiru, Chagres, Punto Toro, Salehabad, Sicilian, Sud An 754-61, Co Ar 3319, Co Ar 4319, Karimabad, Naples, and Pacui. Saint Floris antibody (homologous 1:256) reacted with An B496 at 1:8. In a neutralization

Table 23

Cross-reactions of Isfahan with Chandipura and Piry Viruses by Mouse Neutralization Test*

Virus	Titer log LD_{50}/ml in in normal ascitic fluid	LNI with antibody of:			
		Isfahan	Chandipura	Piry	VS-Indiana
Isfahan	7.1	≥4.9	2.4	1.6	1.7
Chandipura	7.3	3.3	≥5.1	4.2	2.1
Piry	7.9	3.7	3.6	≥6.4	0.8
VS-Indiana	9.3	2.2	0.8	0.3	5.1

*
Suckling mice, inoculated intracerebrally with mixtures of undiluted mouse serum or mouse ascitic fluid and dilutions of virus; Serum-virus mixture incubated 1 hour 37°C; antibody made in mice with 4 inoculations of virus plus Freund's complete adjuvant.

Table 24

HI reactions of Utinga and 2 other Simbu Group viruses from Panama

	Antibody		
Antigen	Utinga	Bradypus 121	Bayano 517
Utinga	≥640	160	320
Bradypus 121	40	≥320	0
Bayano 517	40	160	320

test in mice i.c., Dak An B496 was neutralized 1.1 log by St. Floris antibody and ≥3.5 log by homologous antibody. Gordil (Dak An B496) appears to be different from but related to St. Floris virus.

Aus NT 16701 isolated from Anopheles meraukensis was referred for study by Dr. R. Doherty of the Queensland Institute for Medical Research, Brisbane, Australia. CF tests with 30 grouping fluids were negative as were CF tests of Aus 16701 ascitic fluid (homologous 1:64) with 16 individual antigens of viruses from Australia and Southeast Asia. HA antigen titered 1:2560 at pH 6.0. HI testing with 31 sera of various arbovirus groups was negative. The virus appears to be a new ungrouped agent.

Brazil H 34675 a known group B virus, was isolated by Dr. Oscar Souza Lopes of the Instituto Adolfo Lutz, Sao Paulo, Brazil from brain of a case of Sao Paulo encephalitis. The virus was received at YARU from Dr. C. Calisher. In plaque reduction neutralization tests in VERO cells, H 34675 was not neutralized (80% plaque reduction) by 1:8 sera of Cowbone Ridge, Jutiapa, SLE, Ilheus, MML, Powassan, yellow fever, Modoc, Dengue 2, Dengue 3, Uganda S, West Nile, Kadam, Dakar bat, Wesselsbron, Banzi, Alfuy, Edge Hill, or Japanese encephalitis. Bussuquara was positive at 1:8, not at 1:16. The homologous titer was ≥1:256. In addition it was not neutralized by a group B grouping ascitic fluid from mice immunized with greater than 40 group B viruses. The Sao Paulo encephalitis agent appears to be a new group B virus.

Mosquito transmission experiments

Mosquito transmission experiments with Orungo virus (O. Tomori and T.H.G. Aitken). Orungo is an African orbivirus which has been isolated from naturally infected mosquitoes in the wild as well as from viremic febrile humans.

Experiments were planned to try and demonstrate mosquito transmission in the laboratory. So far viremia has not been demonstrated in several laboratory animals (mice, hamsters, chicks and sparrows) inoculated subcutaneously, although low-grade viremia can be produced in mice and hamsters inoculated intracerebrally (i.c.). Accordingly mosquitoes had to be infected by means of an artificial virus-blood meal or else inoculated with virus parenterally. Likewise, no known satisfactory laboratory animal was available to demonstrate virus transmission and hence in vitro techniques were utilized. These consisted of (1) feeding the isolated infected mosquito on a drop of blood placed on the fine netting covering the mosquito holding chamber, or (2) forced-feeding of the mosquito on blood in a minute glass capillary; subsequently, either type of blood specimen was mixed with a like amount of 2.5% fetal calf serum and inoculated parenterally into a group of 10-15 fresh mosquitoes to facilitate multiplication of any virus which might have been transmitted in the feeding process. The mosquitoes were held 7-10 days at 26.7°C after which they were tested for virus by grinding and inoculating them i.c. into baby mice. Brains of mice

showing typical infections were harvested and tested for virus, which if present, was identified as Orungo by CF test.

Adult female Aedes albopictus and A. aegypti mousqitoes were exposed to Orungo virus. After 6 days, virus was demonstrated in one orally-exposed A. albopictus, but only inoculated mosquitoes (4/10 females) transmitted virus to blood droplets after an extrinsic incubation period of 7-10 days. Mosquitoes had been inoculated each with 1.8 dex per ml of virus. At the time of transmission attempts (6-10 days), virus titers in individual mosquitoes varied from 2.3 to 3.0 dex per ml, thus the virus titer increase ranged from 0.5 - 1.2 dex. Only one parenterally-inoculated A. aegypti transmitted virus. It would be desirable in future experiments to test mosquitoes after longer incubation periods, particularly in the case of the orally-"infected" females.

Development of arbovirus techniques and virus models

Salt-dependent hemagglutination with Bunyaviridae antigens. (B. Beaty, R. Shope, and D. Clarke). Recently, Inaba reported enhanced HA for Akabane virus of the Simbu group of the Bunyaviridae. Unlike antigens of the Togaviridae which commonly have high HA titers after acetone extraction, many Bunyaviridae antigens are poor hemagglutinators. Satisfactory HA antigens are often obtained only after additional manipulation, such as sonication or special culture techniques. The classic methods of Clarke and Casals for HA and hemagglutination-inhibition (HI) specify a 0.15M NaCl viral adjusting diluent (VAD). An initial experiment confirmed the report of Inaba with a Simbu group antigen. We then tried other Bunyaviridae antigens which had low or nondetectable HA titers to see if they would be satisfactory antigens when used with higher salt concentrations.

Representative lyophilized sucrose-acetone extracted mouse brain antigens from 10 serologic groups were used. Some of these antigens had been sonicated. Antigens were reconstituted in water and allowed to dissociate one hour before serial 2-fold dilution in bovine albumin borate saline pH 9. Two LaCrosse virus tissue culture antigens had been tween-80-ether treated by the method of Chappell.

HA and HI were carried out according to Clarke and Casals using goose erythrocytes suspended in phosphate buffers which were adjusted to have final molarities of 0.15, 0.25, and 0.4 NaCl. Titrations were in microtiter plates with antigens diluted either in tubes or by loops. The dilution techniques yielded comparable results. Antigens were used at final pH's 5.75, 6.0, 6.2, and 6.4 except for Congo antigens at pH 6.8, 7.0, 7.2, and 7.4. Four HA units were employed in HI tests.

Antibody as reference sera or ascitic fluids was from mice immunized with from 2 to 4 intraperitoneal injections of infected mouse brain. Antibody preparations were acetone extracted and goose cell absorbed before use.

Table 25 shows the effect of increased NaCl concentration on 29 antigens representative of 10 different serologic groups. Enhancement of HA titer was typically greatest at pH 5.75. Nine antigens had ≥4-fold increase in HA titer in the hypertonic saline. Four of these (Anopheles A, Bwamba, LaCrosse, and Oropouche) had no demonstrable HA at 0.15M NaCl, but were high titered at higher NaCl molarities.

Table 26 demonstrates the specificity of the enhanced agglutinins by HI. In all cases, the agglutination was inhibited by homologous antiserum, and in cases of cross-reaction, the homologous antiserum inhibited to higher titer than the heterologous. Two LaCrosse tissue culture antigens had titers of 1:2048 at 0.4M NaCl, but specificity could not be proven by HI test. These non-specific salt-dependent agglutinins may result from contaminating agents in the cell cultures.

Hypertonic saline had no demonstrable effect on the HA titer of 7 of 29 antigens. Twenty-two of the antigens had at least 2-fold increase in HA. Nine of these were ≥4-fold increases. Sonicated antigens accounted for most (89%) of those with ≥4-fold increases, while none of these without a demonstrable HA enhancement had been sonicated.

Table 27 shows the cumulative effect of increased NaCl and sonication on the same antigen. There was an additive effect of the two treatments resulting in increased HA titer. The LaCrosse antigen was unusable at 0.15M NaCl, but titered 1:32 at 0.4M NaCl after sonication. The Sicilian and Tahyna antigens were functional at 0.15M NaCl without sonication, but the combination of techniques resulted in much higher HA titers.

The mechanism by which high salt concentration increases Bunyaviridae HA titer is not known. Enhancement occurs in NaCl, unlike the measles salt-dependent antigen which functions only in other salts. That some Bunyaviridae antigens in our experiments did not increase in titer at 0.4M NaCl does not mean lack of capability of enhancement since optimum conditions of virus substrate, molarity, pH, and erythrocyte type have not yet been established and may not have been achieved in these experiments. The results do, however, indicate the broad occurrence of the salt dependent hemagglutinin among Bunyaviridae, and the wide applicability of this simple enhancing technique in diagnostic virology.

HA and HI comparison of goose, glutaraldehyde-fixed-goose, and trypsinized human O cells. (D. Clarke). Glutaraldehyde-fixed-goose cells may be stored for up to 1 year and thus may have advantage of economy, availability in the field, and ease of handling over the standard goose cell and the trypsinized human O cell for HA and HI testing of arboviruses. Results of a comparison of the 3 cell types are shown in Table 28. The cell dilutions were: Goose, 1:24 of 8% stock; glutaraldehyde-goose 1:30 of 30% stock; and trypsinized human O, 1:30 of 10% stock. The results indicated that for some antigens, glutaraldehyde treated cells are

Table 25

Effects of increased NaCl molarity on hemagglutination (HA) titers of 29 Bunyaviridae antigens

Antigen	Sonicated	Reciprocal of HA titer at NaCl molarity 0.15	0.25	0.4
Anopheles A	yes	<4*	128	256
Apeu	no	<4	<4	<4
Bunyamwera	no	<4	8	8
Buttonwillow	yes	16	32	64
Bwamba I	yes	<4	64	128
Bwamba II	no	<2	<2	<2
Bwamba II	yes	<2	<2	2
California	yes	2	<2	16
Capim	yes	8	16	16
Congo I	yes	64	32	128
Congo II	yes	<2	<2	4
Guajara	yes	16	64	64
Itaqui	yes	64	64	128
Jurona	no	<2	<2	<2
Keystone	no	<4	<4	<4
LaCrosse	no	<2	<2	2
LaCrosse	yes	<2	4	32
Manzanilla	no	<2	<2	<2
Oropouche	yes	<4	64	128
Sicilian I	no	<4	8	8
Sicilian II	no	32	64	512
Sicilian II	yes	64	512	>1024
Snowshoe hare	no	4	8	8
Tahyna I	yes	4	8	8
Tahyna II	no	16	32	32
Tahyna II	yes	256	128	512
Turlock	no	4	4	2
Trivittatus	no	2	<2	<2
Witwatersrand	no	16	16	32

*Viral adjusting diluents were pH 5.75, 6.0, 6.2 and 6.4 for all antigens except Congo with which diluents of pH 6.8, 7.0, 7.2 and 7.4 were used. Optimal HA pH is not listed, but enhancement was typically greatest at 5.75.

Table 26

Specificity of enhanced HA titers as determined by hemagglutination inhibition

Antigen*	NaCl Molarity	Antiserum				
		Homologous	California (BFS-283)	Pongola	Lukuni	Normal
Anopheles A	0.25	320**	0	0	0	0
Anopheles A	0.4	320	0	0	0	0
Bwamba	0.25	160	0	0	0	0
Bwamba	0.4	320	0	0	0	0
Guajara	0.4	80	0	0	0	0
LaCrosse	0.4	40	0	0	N.D.	N.D.
Sicilian	0.15	80	0	0	N.D.	N.D.
Sicilian	0.4	80	0	0	N.D.	N.D.
Tahyna	0.15	80	20	0	N.D.	N.D.
Tahyna	0.4	160	40	0	N.D.	N.D.

*All antigens were sonicated.

**Reciprocal of highest serum dilution inhibiting agglutination by four units of antigen; 0, no inhibition at 1:10 serum dilution.
N.D. = not done.

Table 27

Effects of sonication and increased NaCl molarity on HA titers of 3 Bunyaviridae antigens

Antigen	Sonicated	NaCl molarity	HA titer (reciprocal)
LaCrosse	no	.15	0
	no	.25	0
	no	.4	2
	yes	.15	0
	yes	.25	4
	yes	.4	32
Sicilian	no	.15	32
	no	.25	64
	no	.4	512
	yes	.15	64
	yes	.25	512
	yes	.4	1024
Tahyna	no	.15	16
	no	.25	32
	no	.4	32
	yes	.15	256
	yes	.25	128
	yes	.4	512

Table 28

Comparison by HA and HI of goose, glutaraldehyde-fixed-goose, and trypsinized human O cells

Antigen	HA			HI (corrected to 4 units)			
	Goose	Glutaraldehyde-goose	Trypsinized human O	Goose	Glutaraldehyde-goose	Trypsinized human O	Antibody
Sindbis	1280	1280	2560	2560	320	10240	mouse ascitic fluid (AF)
Itaqui	256	256	256	1280	1280	1280	mouse AF
EEE	8000	16000	32000	640	1280	5120	mouse AF
Dengue 1	3200	6400	6400	5120	320	1280	mouse AF
Dengue 2	3200	3200	3200	640	40	640	mouse AF
Dengue 3	200	200	1600	1280	640	10240	mouse AF
yellow fever (17 D)	2560	640	5120	160	80	320	human serum
yellow fever (FV)	25600	< 10	25600	640	-	640	human serum
Semliki	80	80	80	2560	5120	10240	mouse AF
KFD	16000	8000	16000	2560	5120	10240	mouse AF

satisfactory, however the HI test in several instances was significantly less sensitive than with standard goose cells or trypsinized human O cells, and the glutaraldehyde-fixed cells are not agglutinated by some group B antigens such as yellow fever and dengue 4 (result not shown in Table 28).

Techniques for study of arenaviruses (J. Casals and D. Budzko). Propagation of arenaviruses in cell cultures. Pichinde and Amapari are being serially propagated in fluid VERO cell cultures; transfers are made between 7 and 10 days after infection of tubes containing cells seeded 4 or 5 days before. The purpose of this work is to determine whether these viruses will develop antigenic variants after a number of such passages, distinguishable by complement-fixation or plaque reduction test from the viruses maintained by newborn mouse brain passage.

Pichinde virus has been transferred 9 times in VERO cultures; CPE was observed up to the 5th passage but not after that. Transfers were made, blindly; following the 9th passage virus was present in the fluid with a titer of $10^{-5.9}$/0.02 ml by intracerebral inoculation to newborn mice.

In a second series of transfers with the same virus, transfers using a pool of undiluted fluid and fluid diluted to 10^{-1} on the one hand, and a pool of fluids diluted 10^{-3}, 10^{-4} and 10^{-5}, it was observed after 4 cell culture passages, that the CPE tended to be less marked with the more concentrated virus than with the diluted one; furthermore, titrations by the ic route in newborn mice showed an LD_{50} of $10^{4.6}$ with the low-dilution-passage virus and $10^{-7.0}$ with the passage using the high dilutions. It is planned to try to use this interfering-like effect of the concentrated virus as compared with its absence from the high-dilution passages to investigate further possible antigenic variation.

Amapari virus has been similarly passaged in VERO cell cultures for 4 consecutive transfers. After at least 6 additional transfers, CF antigens and immune ascitic fluids will be prepared with this virus and compared with the original mouse maintained agent.

Preliminary experiments have been carried out (in association with H. Siebens, 1976) on the separation of virion and sub-virion size particles of Pichinde virus by rate-zonal centrifugation in sucrose gradients. These studies have been undertaken in order to prepare purified fractions that will be used in radioimmunoassays. Fluids from VERO cell cultures in roller bottles infected with the virus were harvested on the 7th post-infection day, virus was precipitated with polyethelene glycol, layered on a preformed gradient and centrifuged. Fractions were collected and tested for infectivity by ic inoculation in newborn mice and by CF test; these two activities could be easily separated.

Cross-reactions among arenaviruses by immunofluorescence. Antigenic relationships among arenaviruses have been investigated in the past, in this laboratory mainly by CF and agar gel diffusion and precipitation tests, and here as well as in other laboratories by neutralization and plaque reduction tests. The increasing application of immunofluorescence (IF) to serological diagnoses prompted our interest in the quantitative examination in that test between the different available arenaviruses.

1. Immune sera and ascitic fluids were prepared in mice by giving them 3 immunizing intraperitoneal (IP) injections of a virus, 0.3 ml of a 10^{-1} suspension, on days 1, 20 and 35, respectively; the mice were bled 7 days after the last injection. When AF's were desired, the immunized mice were given an IP injection of mouse sarcoma 180, 7 days prior to paracentesis. A few human sera from persons who had recovered from Lassa fever, LCM, and Argentinian hemorrhagic fever were available and used in cross tests.

2. Antigen-containing preparations for Lassa virus were generously supplied by CDC, Atlanta; they consisted of suspensions of VERO cells infected with the virus, inactivated by exposure to ultraviolet light, and deposited in the form of drops on teflon-coated slides on which 12 circular spots about 5 mm in diameter had been left uncoated.

Various types of antigen-containing slides for other arenaviruses have been prepared in this laboratory: chamber-slides (Lab-Tek Products, Division of Miles Laboratories, Inc.) on which BHK-21 or VERO cell cultures were infected with LCM, Tamiami, Tacaribe, Amapari or Pichinde viruses; VERO cell fluid cultures infected with LCM, deposited on slides in the form of drops; mouse brain tissue smears or suspensions infected with LCM; and cell suspensions from ascitic fluids of mice bearing mouse sarcoma 180, infected with LCM or Pichinde viruses.

3. The technique used for IF was as follows: infected monolayers on chamber slides were thoroughly washed with phosphate buffered saline, fixed by immersion in acetone for 10 minutes and stored at -60°C until used. Drops on spot-slides were first dried at 37°C for 15 to 20 minutes then fixed as above. Commercial conjugates (fluorescein isothiocyanate) were employed; indirect type IF was used and an incident light Vanox Olympus microscope set for blue fluorescence, with a Xenon light source were employed.

4. Quantitative results of cross-reactions utilizing samples of the same immune sera and ascitic fluids for all the tests are shown in Table 30. The study is far from completed as yet; the results so far indicate that viruses such as Tamiami, LCM and Lassa, which by CF were but distantly related to the other viruses of the family and by plaque reduction test completely unrelated, were easily shown to share antigens by IF. In particular, the reciprocal cross-relationship between LCM and Lassa viruses were easily demonstrable by this test. An additional important observation bearing on the serological identification of

arenaviruses was that a Tacaribe-complex grouping ascitic fluid, prepared by immunizing mice with Amapari, Junin, Tacaribe, Pichinde and Tamiami viruses reacted strongly with LCM and Lassa viruses as well as with the agents in the immunizing schedule; end-points have not as yet been determined but the quality and intensity of the fluorescence observed in the reactions with LCM and Lassa viruses were excellent.

The extent of cross reactions by IF with human convalescent sera is illustrated by the results on Table 29. It is evident that some cross reactions can be strong enough, when compared with the homologous ones, to present diagnostic difficulties.

Experimental Junin viral disease in mice as a model for tolerance to arena viruses. (D. Budzko and C. Clark) Protection of Junin-infected newborn mice by injection of Corynebacterium parvum: Treatment with killed Corynebacterium parvum causes a variety of immunologic phenomena varying from immunosuppression to adjuvanticity, including a powerful stimulation of the reticuloendothelial system. The effects depend upon the experimental conditions employed. We used Corynebacterium parvum to explore the mechanisms involved in the pathogenicity of Junin virus.

Random-bred, Charles River CD (R-1) new-borne mice were injected i.c. with 1000 LD_{50} of the XJ strain of Junin virus. Untreated, infected animals died between the 11th and 18th day after inoculation. The suspension of Corynebacterium parvum (CP) was administered intraperitoneally in different doses. As shown in Figure 1 , administration of killed Corynebacterium parvum on the day of birth, less than one minute prior to intracerebral injection with Junin virus, resulted in a marked enhancement of resistance to infection as evidenced by both increased survival and increased survival time. Sixteen mice were used in each group. The survivors were alive and apparently healthy at 360 days after infection. This protective effect of CP was dose dependent, with maximal protection obtained with a dose of 280 µg of CP per gram of body weight. CP-protected mice showed only mild signs about the 10th day after infection; they did not become paralyzed and recovery was complete by 20 days after infection.

The distribution and localization of lesions in the brains of the Junin virus-infected mice resembled that produced by other Arenaviruses such as Tacaribe virus in the new-born. Although the neuroparenchyma was heavily infected, necrotic foci were rarely seen outside the cerebellum. Histological examination of the brain tissue of mice killed 9, 11, 13 and 16 days after infection, showed zones of infiltration of vascular walls with inflammatory cells and perivascular accumulation of mononuclear cells, and in a few areas, a lesser neuron density with moderate satellitosis. The lesions occurred throughout the acute phase of the disease, although vascular infiltration was seen only after the 11th day of infection. The cerebellum showed numerous areas lacking Purkinje cells. The distribution and localization of the brain lesions of

Table 29

Cross-reaction by immunofluorescence between LCM and Lassa fever convalescent human sera

Patient	Infection	Antigen	
		Lassa	LCM
LP	Lassa fever	256*	8
JC	Lassa fever	32	8
SA	Lassa fever	64	16
WT	Lassa fever	64	16
NF	Lassa fever	16	8
DE	Lassa fever	128	5+
NL	Lassa fever	8+	5+
RC	LCM	16	128

*Reciprocal of serum titer.

Table 30

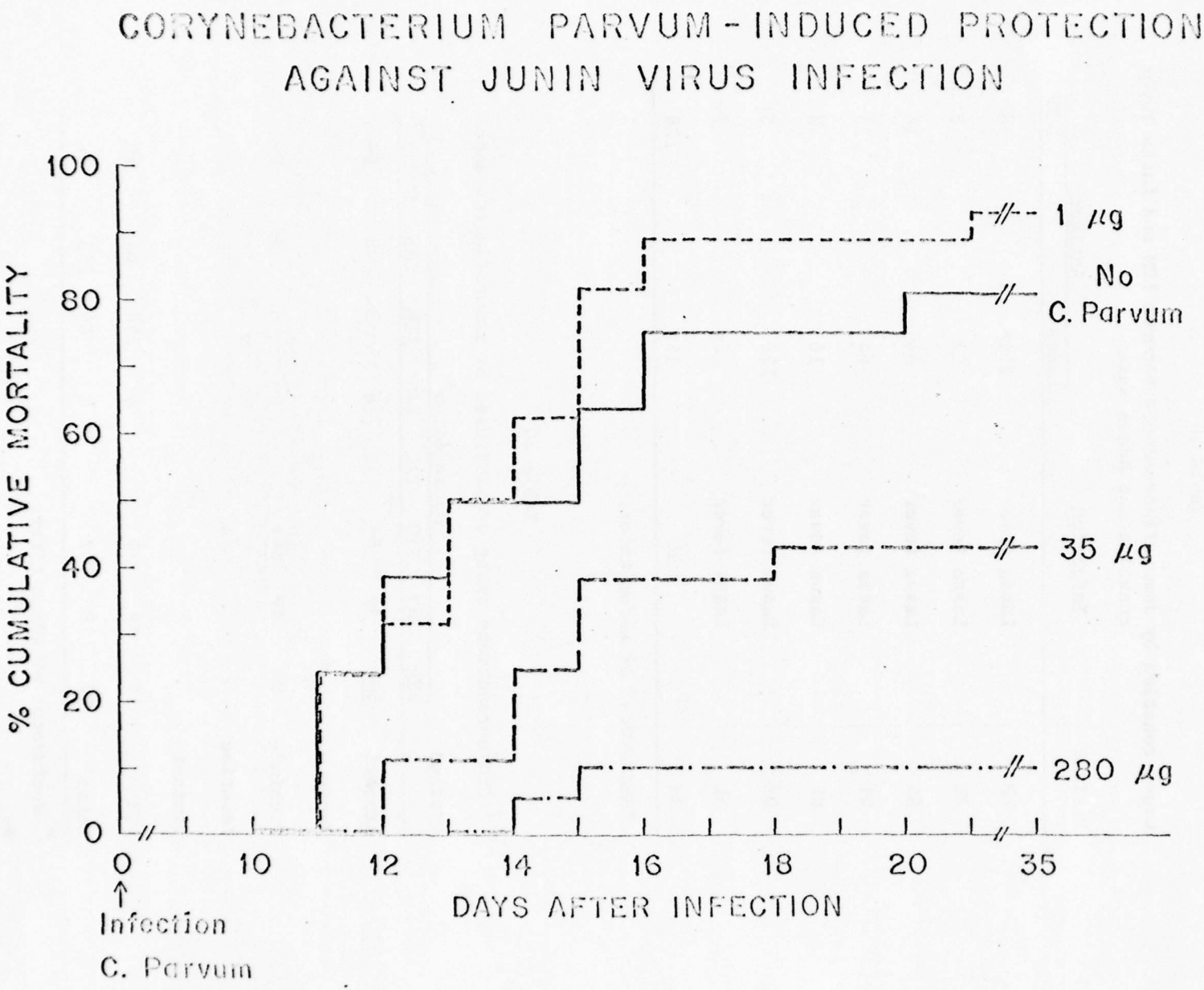
Cross-reactions among arenaviruses by immunofluorescence

Antigen	Mouse serum*							
	AMA	JUN	PIC	TAC	TAM	LCM	LAS	Group**
Amapari	64+	8+	8+		8	<8	<8	8+
Junin								
Pichinde	8+	8+	64+		8+	8	8+	8+
Tacaribe								
Tamiami								
LCM	16	<4	8		8	256	64	8+
Lassa	32	8	32		8	64	256	8+

* Reciprocal of serum titer.

** Polyvalent Tacaribe complex immune ascitic fluid from mice immunized with: Amapari, Junin, Pichinde, Tamiami and Tacaribe viruses.

Figure 1


CORYNEBACTERIUM PARVUM - INDUCED PROTECTION
AGAINST JUNIN VIRUS INFECTION
% CUMULATIVE MORTALITY
100
80
60
40
20
0
1 μg
No
C. Parvum
35 μg
280 μg
0
10
12
14
16
18
20
35
DAYS AFTER INFECTION
Infection
C. Parvum

Corynebacterium-treated, infected mice were the same as untreated mice. As shown in Figure 2 , CP, in doses of 280 µg per gram on body weight basis, given 3 days after infection still had a protective effect and one of lower magnitude was obtained when the interval was extended to 6 days. No protective effect was observed when CP was given 7 or more days after infection.

Administration of CP prior to viral inoculation constituted a less effective protective sequence. Figure 3 top, shows that very little protection was afforded when CP was given only one day prior to infection and the lower panel shows that no effect was obtained when CP was given 3 days prior to infection.

The mechanisms by which CP may alter the course of an infection are unknown. To gain a further insight we measured virus and antibody titers in both CP-protected and untreated animals.

The viral and antibody titers measured by complement fixation and neutralization are shown in Table 31. The mean values of the viral titers of two separate brains expressed per .02 grams of brain of CP-protected mice did not differ significantly from those of the control.

Cerutti (1) showed that CP does not stimulate interferon production in mice. Consistent with her finding, interferon seemed to play no role on CP-induced protection of mice against Junin virus infection. We did not find less virus in the CP-treated animals as would have been expected with an interferon effect. These similar viral titers would also exclude an indirect effect of CP on either the virus or virus-infected cells. The findings are consistent with the hypothesis that Junin virus disease in the mouse is associated with host reaction to the presence of the virus rather than the consequence of simple viral replication and direct cell destruction.

Infant mice born from CP-protected, virus-infected parents, showed high titers of virus in their brains when tested 24 hours after birth, indicating that viral infection in uterus had taken place. These infants resisted a challenge with 100 LD_{50} of virus inoculated intracerebrally.

The possibility that CP acted through its known adjuvant capacity seems unlikely since CF antibody titers in serum, shown in Table were equally negative in both groups during the first 11 days of the disease and antibody titers in the two groups during the acute stage of the disease did not differ significantly. Appearance of the NT antibodies also occurred at the same time in both groups and with a similar frequency. We found high titers of serum neutralizing antibodies in adult CP-protected mice concomitant with high titers of virus in their brains.

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(1) Cerutti, I. Antibiral properties of Corynbacterium parvum. In "Corynbacterium parvum, application in experimental and clinical oncology. Ed. by B. Halpern. Plenum Press, New York, 1975.

Figure 2

THE TIME EFFECT OF CORYNEBACTERIUM PARVUM ON JUNIN VIRUS INFECTION IN BABY MICE

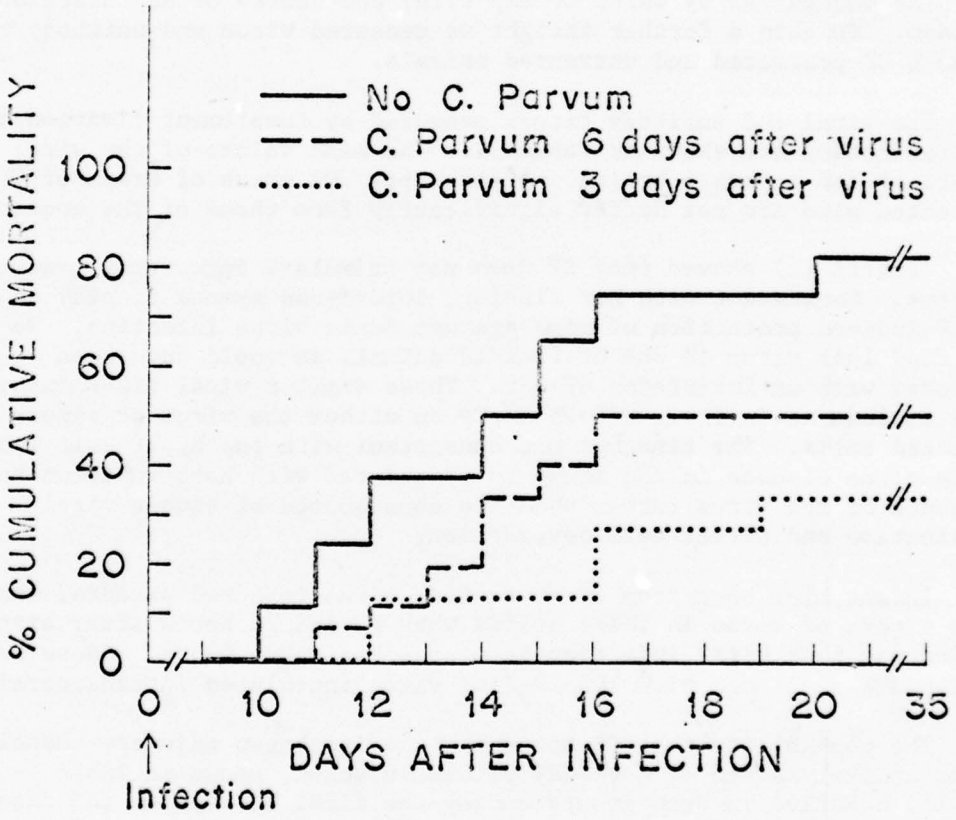

Figure 3

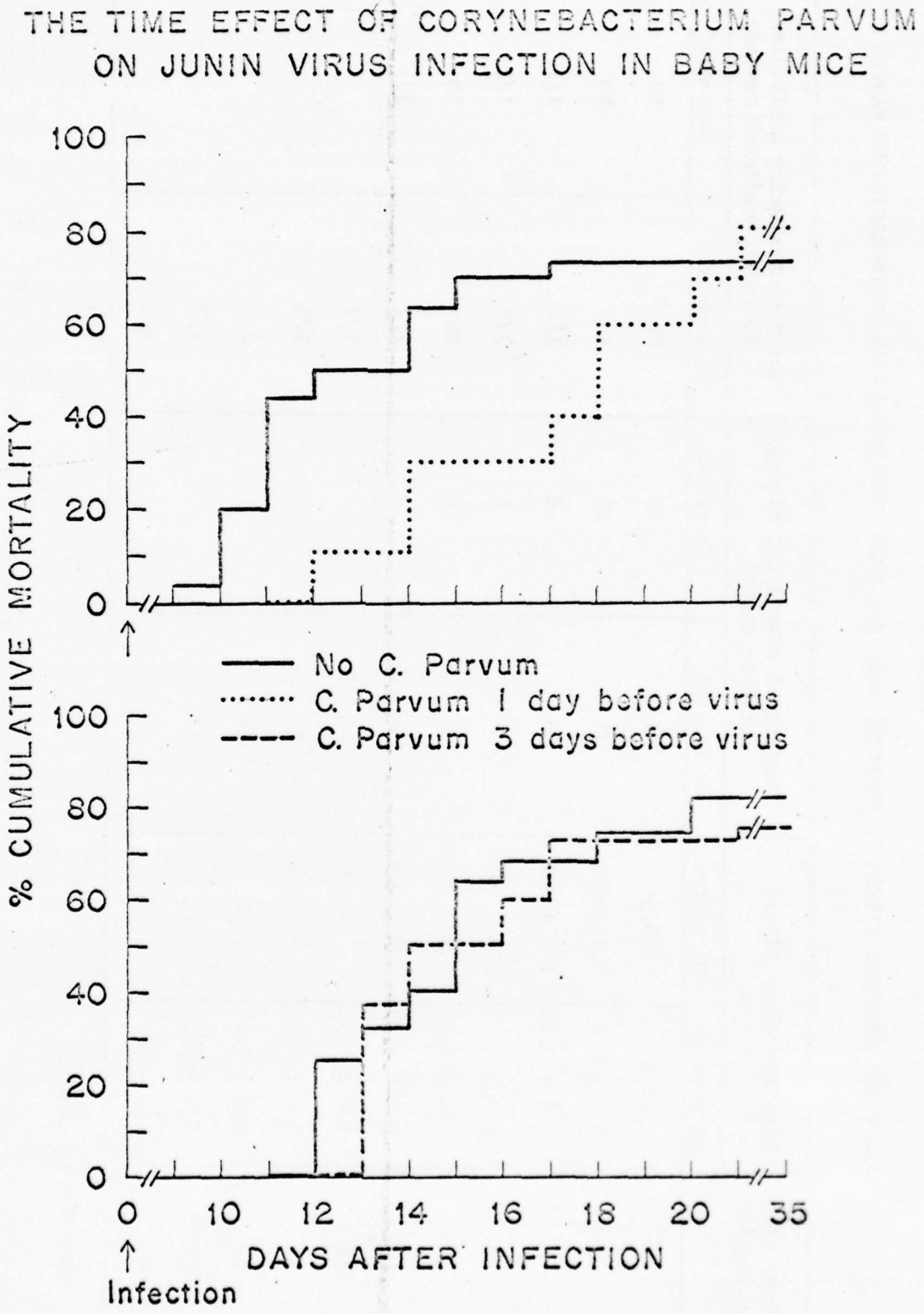
THE TIME EFFECT OF CORYNEBACTERIUM PARVUM
ON JUNIN VIRUS INFECTION IN BABY MICE
% CUMULATIVE MORTALITY
100
80
60
40
20
0
No C. Parvum
C. Parvum 1 day before virus
C. Parvum 3 days before virus
0
10
12
14
16
18
20
35
DAYS AFTER INFECTION
Infection

Table 31

VIRUS AND ANTIBODY TITERS IN INFECTED MICE FOLLOWING TREATMENT WITH CORYNEBACTERIUM PARVUM

Days after infection	Mean log virus titers ± S.D.		Reciprocal complement fixing antibody titers		Serum neutralizing antibodies (No.positive/Total tested)	
	CP Treated	Untreated	CP Treated	Untreated	CP Treated	Untreated
2	<0.5	<0.5	ND	ND	ND	ND
3	3.0 ± 0.1	3.1 ± 0.3	ND	ND	ND	ND
7	7.2 ± 0.1	6.5 ± 0.6	0	0	1/2	1/2
9	6.1 ± 0	6.4 ± 0.6	0	0	2/2	1/2
11	6.0 ± 0	5.9 ± 0	0	0	ND	ND
16	6.6 ± 0.7	5.8 ± 0.4	0	8	1/2	1/2
28	5.0 ± 1.2		16		1/2	
62	3.0 ± 0		32		1/2	
75	5.0 ± 0.2		32		2/2	
120	5.1 ± 0.5		32		2/2	
140	4.1 ± 0.8		64		2/2	

ND = Not Determined

Protection by Corynebacterium parvum may be due to a cell-mediated phenomenon. In associating the protection induced by C. parvum with its known cellular immunological properties, the logical cell candidates appear to be either macrophages, known to be activated by C. parvum, or T cells, whose function has been shown in several instances to be suppressed by CP.

We have investigated the possible role of the macrophages on the course of the infection, using a basically selective macrophage poison, crystalline silica. Figure 4 shows that silica particles, administered intraperitoneally (0.2 mg per gram body weight) either 24 or 4 hours prior to infection, systematically induced a state of enhanced resistance. These data seem to be inconsistent with the possibility that CP causes its protective effect through in vivo activation of macrophages. In addition, it would be difficult to explain the lack of effect of CP in some of the sequences tried, on the basis of macrophage activation alone. Weissenbacher et al. were able to demonstrate that neonatal thymectomy prevented the onset of Junin viral disease in new-born mice (1). As in our case, they found that the titers of brain virus did not differ from those of sham-thymectomized, infected mice. CP is known to cause a marked decrease in the size of the thymus in adult mice and if this were the case in the new-borne also, it might cause a "functional" thymectomy. C. parvum, on the other hand, produces depression of T-cell mediated immunity in general and thus, might interfere with some of the immune events associated with the pathogenicity of Junin virus.

As to the participation of the T-cells in the CP protection no conclusive results have been obtained. Attempts to perform experiments with inbred new-born mice, which are absolutely necessary in cell transfer experiments, have been handicapped by the following: a) the virus must be inoculated within the first 24-48 hours of life. Manipulating the babies irritated the mothers, and not many of the new-born survived. b) although we obtained fairly good results infecting the new-born from C3HeB/FeJ mice, these babies were very sensitive to CP and only CP in very low doses (<1 µg) was tolerated. The participation of the T cell in the pathology of experimental Junin virus disease in mice was therefore approached in a different way, described in the next sections.

Studies on lymphocytes of Junin virus infected mice. The interaction between the immune system of guinea pigs and Junin virus was studied by Parvdi and colleagues (1). They demonstrated immunosuppressive effects such as decreased primary and secondary responses

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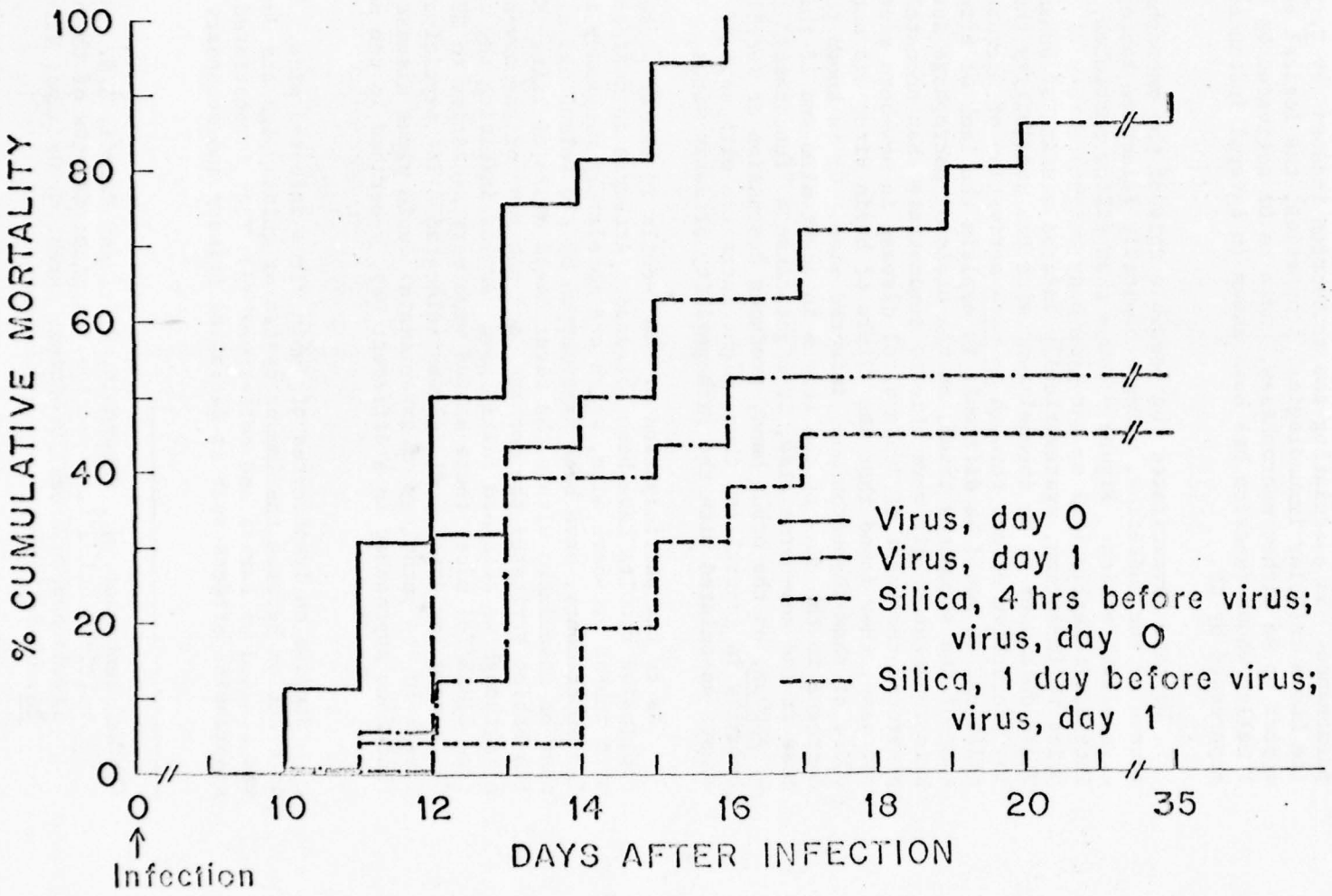
(1) Weissenbacher, M., Schumunis, G.A., and Parodi, A.S. Junin virus multiplication in thymectomized mice: Effects of thymus and immunocompetent cell grafting. Arch. f. Gesampt. Virusf. 26: 63-66, 1969.

Figure 4

THE EFFECT OF SILICA ON JUNIN VIRUS INFECTION IN BABY MICE



to particulate antigens (red blood cells) and decreased numbers of antibody forming cells and circulating hemagglutinins. In order to clarify further the role of the lymphocyte in CP induced protection and study the participatin of lymphocytes in the pathogenesis of Junin virus disease in mice, we undertook the following experiments:

1. Response of spleen lymphoid cell cultures to phytohemagglutinin (PHA), conconavalin A (con A) and endotoxin lipopolysaccharide from E. coli (LPS) from infected and non-infected infant and adult mice.

2. Mixture of infected lymphoid cells with non-infected followed by stimulation by PHA, con A and LPS in order to establish the role of suppressor cells in the system.

3. In vitro infection with Junin virus of cells of the lymphoid system and further stimulation of DNA synthesis by mitogens to rule out the participation of suppressor cells in the impaired response obtained from cells of infected animals.

Results:

1. To study the response to mitogens of the spleen cells of infected mice the C57Bl/6J strain was employed. Infant mice (24 hrs. of age) were inoculated intracerebrally with 1000 LD_{50} of Junin virus. Only 35% of the mice died between 12-17 days after inoculation. The remaining animals did not show signs of disease and survived more than 3 months. They carried virus in their brains for more than 2 months (titers: $>10^{-3}$ LD_{50}; determined by complement fixation test and cytopathogenic effect on Vero cells).

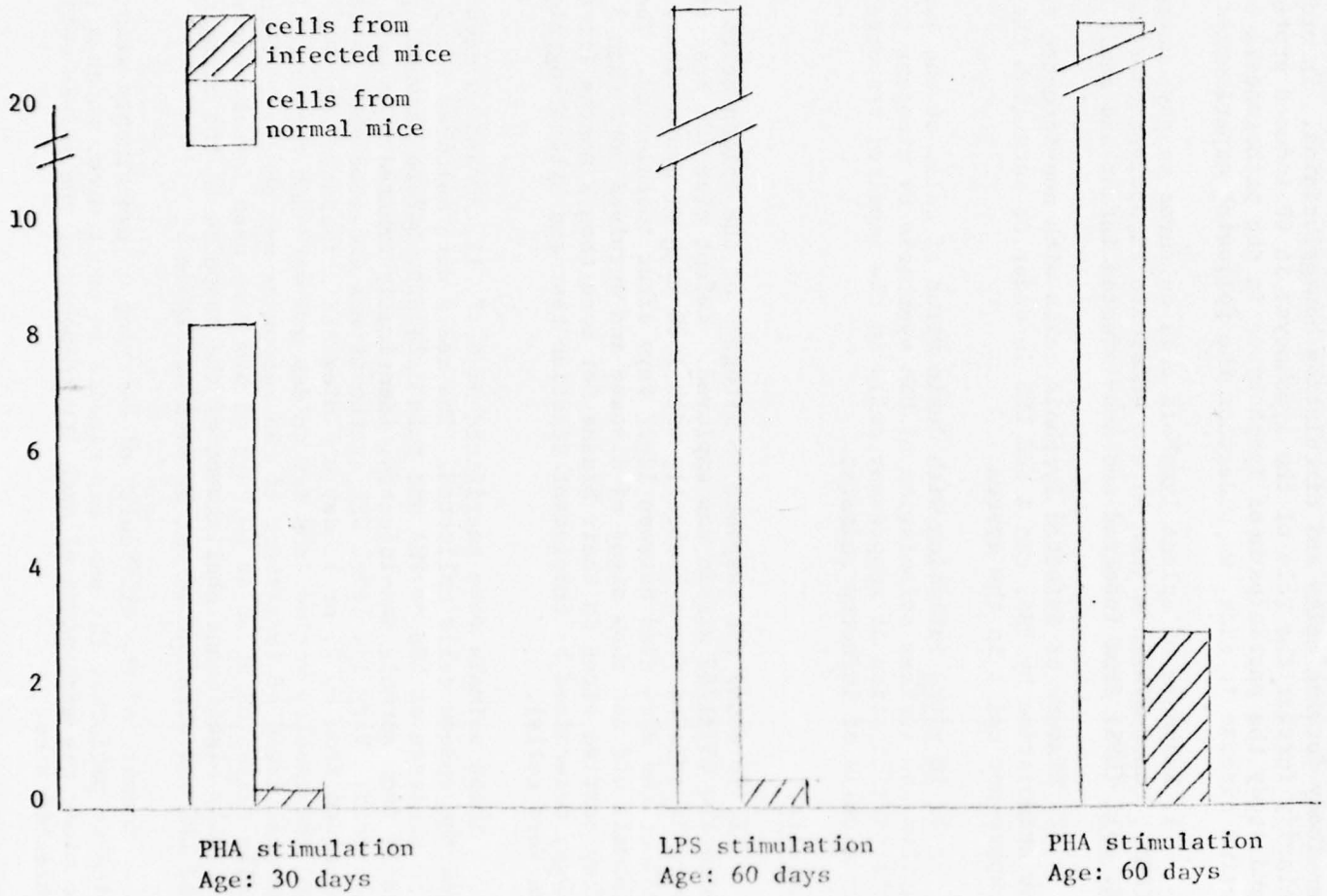
These animals were sacrificed at 6, 7, 11, 30 and 60 days of age and the spleen cells collected. The cells were cultured for 3 days in the presence of LPS or PHA and pulsed 24 hrs. before harvesting. Spleen cells from normal, non-infected, identically treated mice were used as controls. Very low level DNA synthesis was detected when cells were obtained from 6, 7, or 11 day old mice and stimulated with PHA or LPS, and the results varied from day to day and were not reproducible. A general trend of impairment of the responses was observe. When spleen cells collected at 30 or 60 days of age were used for the experiments a highly significant abolishment of the response to the mitogens PHA and LPS was obtained as can be seen in Figure 5.

Because of the difficulty of carrying on experiments with new-born mice we performed the next experiments in adult mice, which allowed us to study the mechanisms of such low response of the spleen cells from infected mice.

For these experiments we inoculated the virus intracerebrally when mice were 6 weeks old and collected the spleen cells 2, 4, 6 and 8 days post-infection. Ninety-five % of the inoculated mice survived the viral infections showing virus in their brain only from days 5 to 10. In the

Figure 5

DNA-synthesis of spleen cells from normal and infected C57B1/6J mice (30 or 60 day old) stimulated with PHA and LPS



same series on days 5 and 7 antigen was detected by immunofluorescence in approximately 30% of their spleen cells.

The results of PHA, Con A and LPS stimulation of the spleen cells of normal and infected adult mice from three different strains (C57Bl/HeJ, C_3HeB/FeJ, and C57Bl/6J) are shown in Figures 6, 7, and 8.

An impairment of the response to mitogens is also observed with adult mice but its nature is transient and full recovery occurs by 8-10 days post infection. The effect on DNA synthesis was noted in all three strains of mice studied.

2) Suppressor cells are not responsible for the impairment of DNA synthesis after mitogenic stimulation of lymphoid cells. Five X 10^5 normal spleen cells from adult mice were cultured with 2.5 X 10^5 spleen cells from infected animals of the same strain (either C57Bl/6J or C3HeB/FeJ) in the prescene of PHA, LPS or Con A. No significant inhibition of DNA synthesis was observed in these cell mixtures in comparison to cultures of 7.5 X 10^5 normal spleen cells from the same strain (Fig. 9). A small expected inhibition was attributed to the number of infected cells that did not respond normally as expected from results of the previous experiment.

3) Further insight into the capacity of the virus to participate in the impairment of DNA synthesis by the cells of the lymphoid system was sought by culturing normal spleen cells with virus in vitro and studying the effect of the virus on mitogen stimulation. Figure 10 shows that cells from normal animals when infected in vitro with Junin virus are much less responsive to LPS stimulation indicating a direct action of the virus on the cells of the lymphoid system, and further ruling out the possibility that suppressor cells could be blamed for the phenomenon. Con A or PHA were used.

Alterations of immunocytes and/or immune regulator cells by virus infection of these cells. Immune dysfunction and production of disease could be attributed to Junin virus (see prior sections). Along with experimental data mentioned above, observations in the laboratory raised some provoking questions. Junin infected new-born mice were noted to have enlarged appearing spleens, weight loss and/or dwarfness. These signs were reminiscent of runt-disease or graft-versus-host (GVH) reaction that occurs when parental lymphocytes are injected into new-born F_1 mice. LCM virus has been associated with a transient form of runting disease. The hypothesis to be tested was that Junin virus caused lymphocyte alterations with attack against self-antigens, i.e. a graft-vs.-host-like reaction. One could infect mice with the virus, remove lymphocytes and add to normal syngeneic lymphocytes to see if there was a GVH-like reaction i.e. a positive mixed lymphocyte cytotoxicity (MLC).

Figure 6 Inhibition of DNA synthesis in spleen cells from infected adult mice by stimulation with Con A

x — x C57B1/6J strain

o — o C3H/HeJ strain

* — * C3HeB/FeJ strain

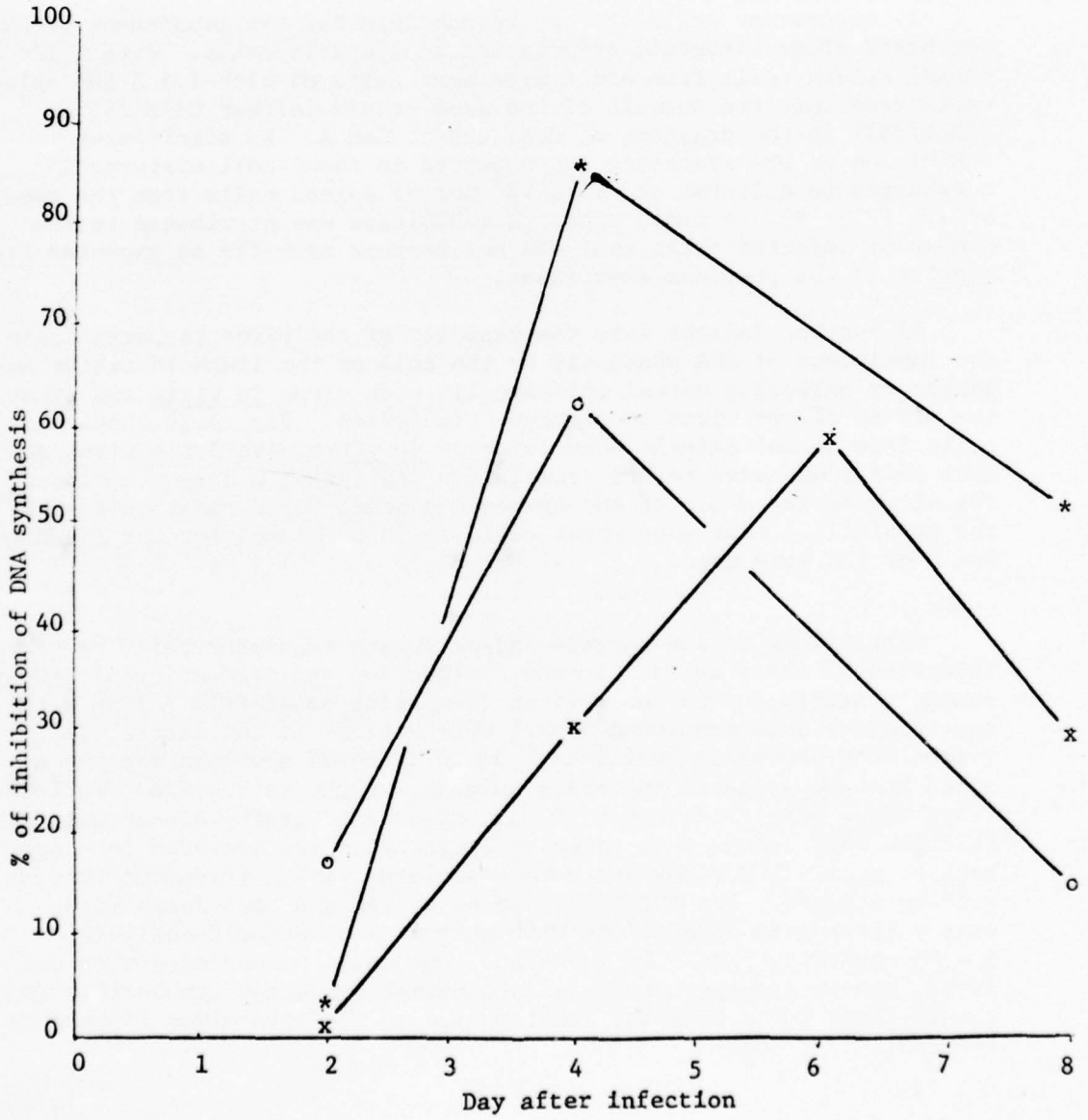


Figure 7 Inhibition of DNA synthesis in spleen cells from infected adult mice by stimulation with LPS

x —— x C57Bl/6J strain

o —— o C3H/HeJ strain

* —— * C3HB/FeJ strain

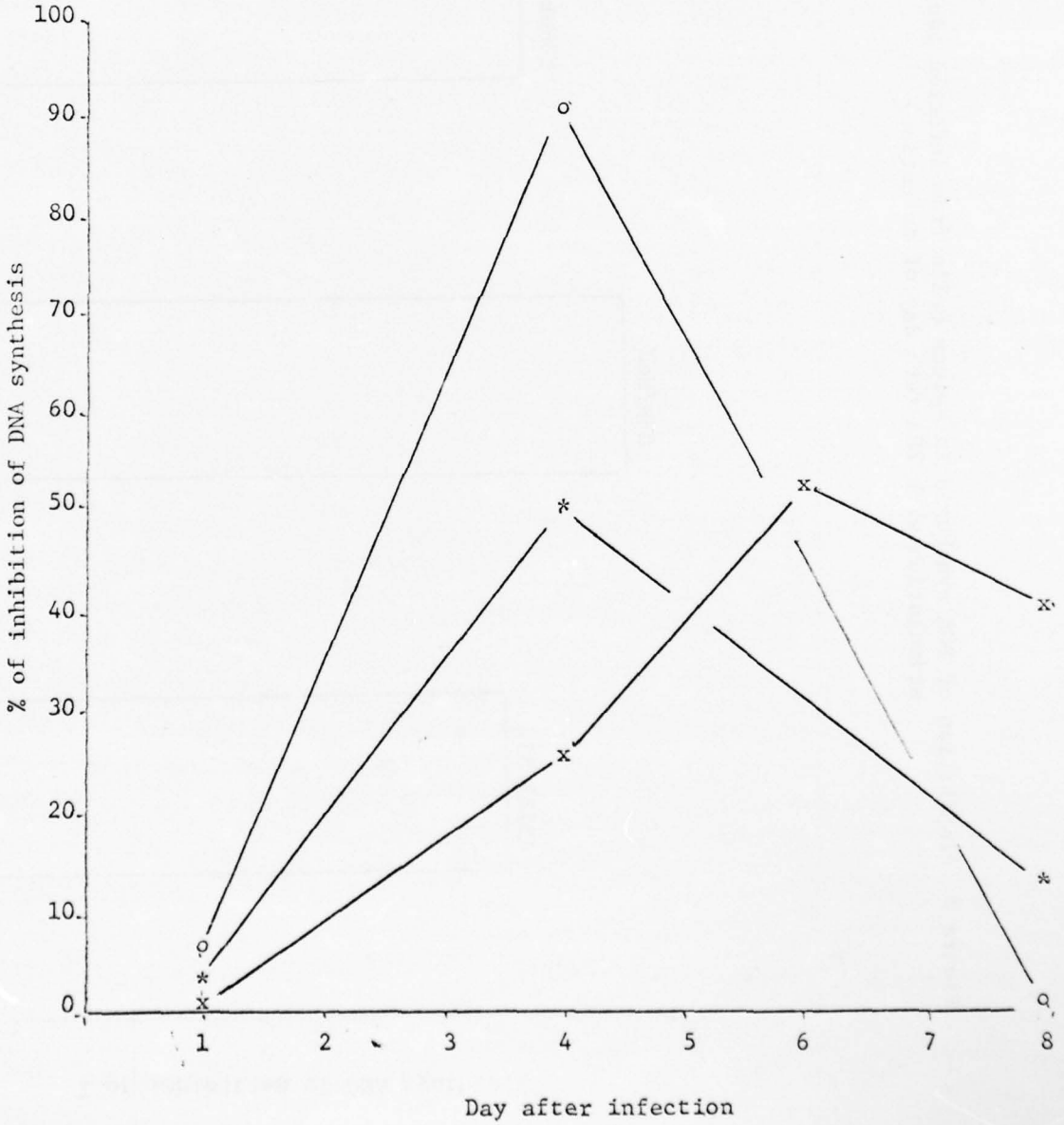


Figure 8 Inhibition of DNA synthesis in spleen cells from infected adult mice by stimulation with PHA (4th day of infection)

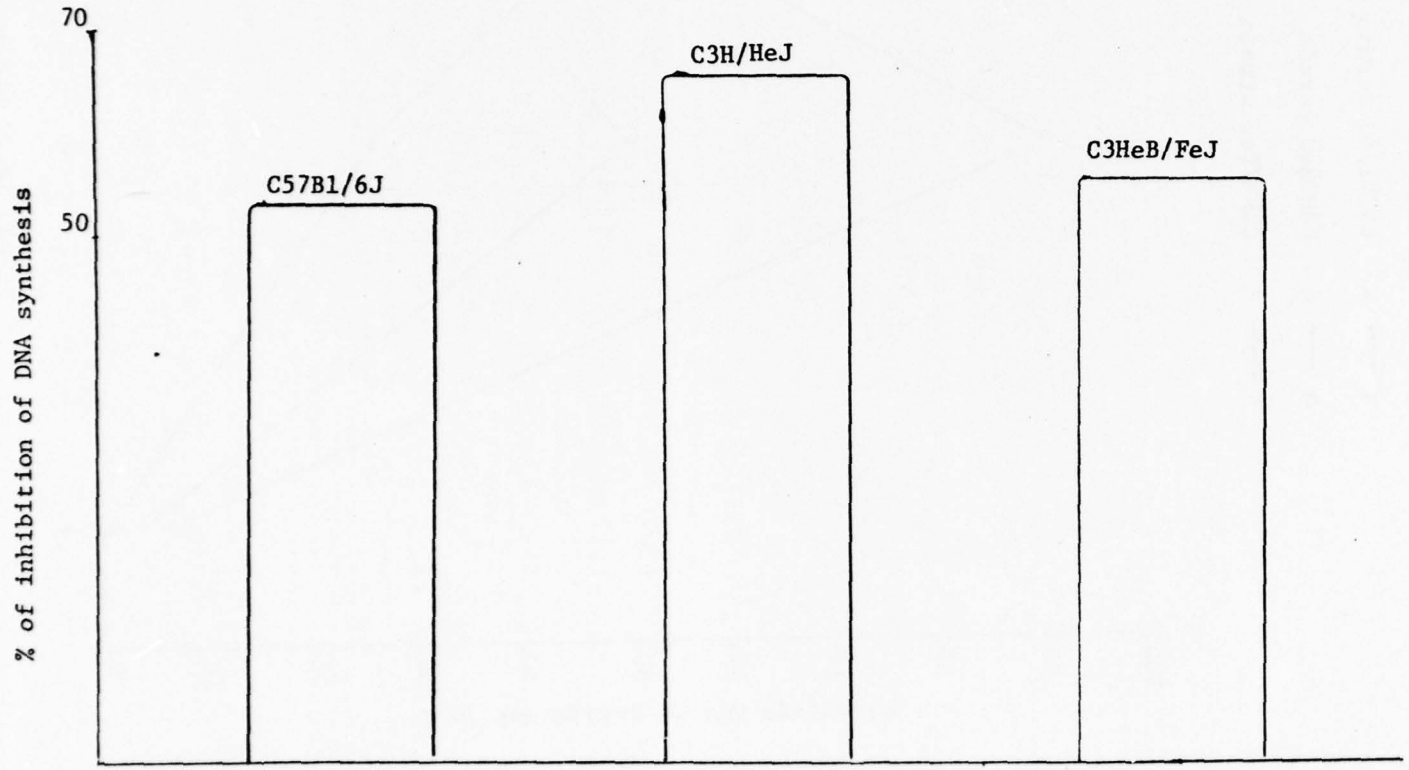


Figure 9 Lack of suppressor activity in spleen cells from C3HeB/FeJ infected adult mice (5th day of infection)

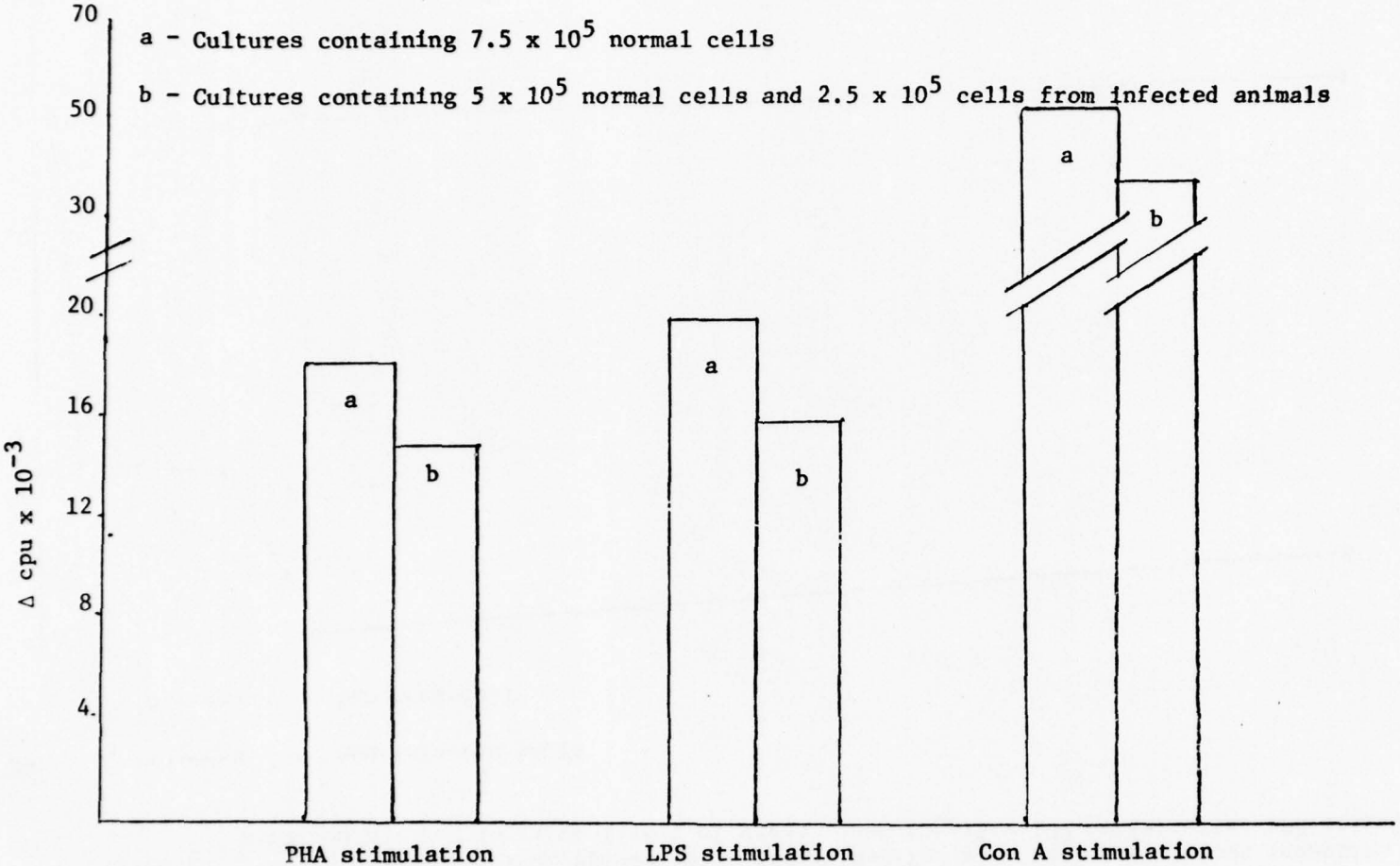
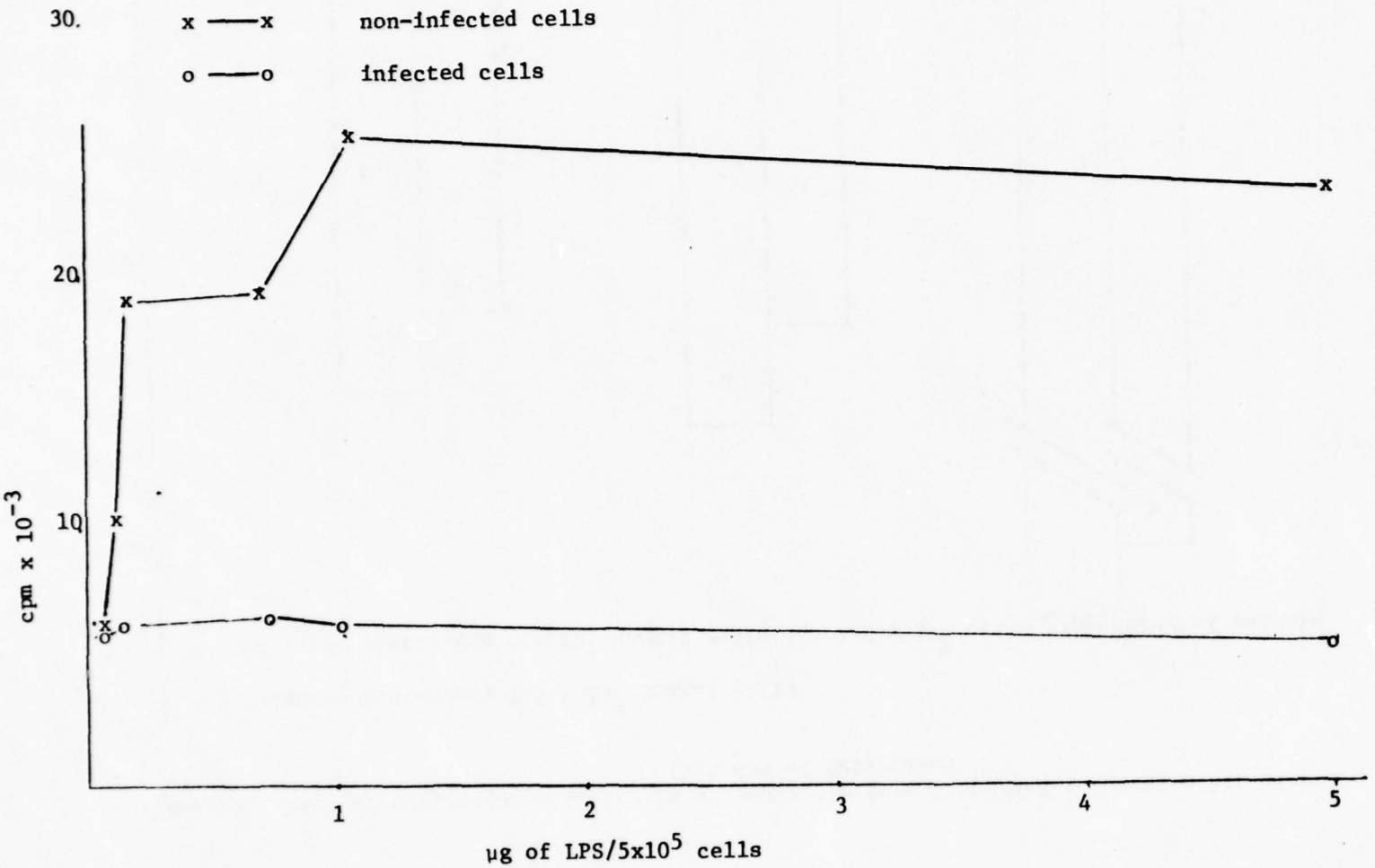


Figure 10 DNA synthesis of normal spleen cells from C3HeB/FeJ mice with and without infection with Junin virus <u>in vitro</u> (Input of virus: 5000 LD_{50}/5 x 10^5 cells) after LPS stimulation



The following experiments were performed:

1. a. MLC with allogeneic infant mice, removing adherent cells if necessary.

   b. MLC between infant-infected and normal syngeneic mice.

2. MLC in adult mouse systems.

No positive MLC was obtained with the allogenic system from infant mice. Since Ly 1,2,3 lymphocytes do not give a positive MLC reaction, the lack of MLC reaction may be due to a preponderance of Ly 1,2,3 cells over other types. Similar results were obtained with the syngeneic system of infected and non-infected infant mouse cells.

In the adult syngeneic system, the results are highly suggestive of a GVH-like-reaction taking place during the temporary disease in adult mice as shown here:

Stimulation indexes between C3Heb/FeJ spleen cells of infected and non-infected adult mice

Days after infection	1	2	4	7	8	9	11
Stimulation index	3.49	.89	.94	2.16	1.27	1.04	1.04

Infected mouse sarcoma cells as antigen for immunofluorescence (J. Casals). Following inoculation of some arboviruses by the intraperitoneal route to mice bearing sarcoma 180/TG, these animals develop high-titered hemagglutinating (HA) and complement-fixing (CF) antigens in the ascitic fluid. Preliminary evidence indicated that the virus replicated in the sarcoma cells since when the virus was inoculated to mice whose ascites was induced by Freund's adjuvant no antigen developed.

The present study was prompted by the desire to increase the number of current methods available for investigation of serological reactions with Congo-Crimean hemorrhagic fever (CCHF) virus, specially to apply to this virus the IF technique. Since replication of CCHF virus with CPE in most cell fluid cultures was not evident, and since previous information obtained in this laboratory had shown that the infective titer of the virus in ascitic fluid reached an LD_{50} of $10^{-5.8}$/ 0.02 cc on IC inoculation to newborn mice and antigenic titers of 1:512 by CF, ascites cells obtained by paracentesis were tried as source of antigen in the IF technique.

Antigen. Groups of 8 or 10 mice, 40 to 60 days old, were intraperitoneally inoculated 0.1 ml of mouse sarcoma 180/TG fluid, on day 1; on day 7 or 8, when ascites was beginning to show, CCHF virus was

inoculated by the same route in an amount of 2 cc of infected newborn mouse tissue, diluted 10^{-1} in saline; this amount of tissue contained between $10^{7.0}$ and $10^{8.0}$ LD_{50}/ml.

On days 4, 5, 6, 7 and if mice survived also on day 8, infected ascites was collected by IP puncture. Three ml of acid-citrate-dextrose was placed in a syringe into which was next aspirated 3 ml of ascites; the procedure was repeated with another mouse and the two tappings were combined. To the pool was added phosphate buffered saline to a total volume of 30 ml and the sarcoma cells were sedimented by centrifugation at 1500 rpm for 10 minutes. The sedimented cells were washed twice in PBS, 30 ml, and again sedimented; after the second washing the packed cells, which had a volume between 1 and 2 ml, were diluted in 3 times their volume of PVS, this constituting the "packed cells". The number of cells per ml was counted using a hemacytometer, and the dilution factor of the packed cells determined which yielded 3×10^6 cells/ml.

Slides for IF tests were prepared employing teflon-coated slides on which 12 circular spots about 5 mm in diameter have not been coated; these slides are supplied commercially. On each spot was deposited one drop through a gauge 27 needle, which drop had a volume close to 0.01 ml; hence each spot contained about 3×10^4 cells. The drops were allowed to evaporate at 37°C, for 15 to 20 minutes, fixed by immersion in acetone at 4°C for 10 minutes and stored at -60°C until used.

Staining and observation of fluorescence. Staining of slides was done by the indirect method; thus far, only mouse and human sera have been tested. The anti-mouse and anti-human gammaglobulin conjugates are purchased commercially; the instrument used in an Olympus Vanox microscope with incident light derived from a Xenon light source, set for blue fluorescence.

Results. Several exploratory tests done with CCHF mouse immune serum at dilution 1:8 only, and a similar serum immune to dengue 2, showed that ascites obtained on days 5, 6, 7 and 8 after inoculation of CCHF virus were specifically positive.

A test was carried out to determine whether antigen would be present at an earlier date, as well as to determine the possibility to quantitate the serum titer, and to investigate whether a cross reaction with CCHF antigen would be observed with a Hazara serum in this type of IF; the result of the test is shown in Table 32.

No antigen was present, at least not in sufficient amount, when the sarcoma cells were collected 4 days after inoculation of the virus, while on the 5th day, and from the preliminary tests described above, on the following ones, antigen was detected; the table also illustrates: the high titer given by a CCHF virus mouse immune serum; the existence by this test of a cross reaction between a Hazara immune serum and the

Table 32

Immunofluorescence using mouse sarcoma cells infected with CCHF virus as antigen

Serum, dilutions		Cells collected on day 4	Cells collected on day 5
CCHF	1:4	0	+
	8	0	+
	16	0	+
	32	0	+
	64	0	+
	128	0	+
Hazara	1:4	0	+
	8	0	+
Dhori	1:4	0	0
Dengue 2	1:4	0	0
None, conjugate only		0	0
None, diluent only		0	0

CCHF antigen, with a serum titer at least 1:8; and the total lack of non-specific IF by unrelated sera even at dilutions as low as 1:4.

The technique of using mouse sarcoma infected cells first developed with CCHF virus has now been successfully applied to LCM and Pichinde virus; it is planned to investigate it for a large number of arboviruses and arenaviruses. If generally successful, the method recommends itself for its simplicity, economy, for its potential as a means for quantitative antigen and antibody studies, and for use in seroepidemiological surveys.

Ixodid tick tissue culture (S. Buckley) A Rhipicephalus appendiculatus tick cell line, TTC-243, established by Dr. M.G.R. Varma and his colleagues Mary Pudney and C.J. Leake (J. Med. Ent. 11, 698-706, 1974) was sent by messenger to YARU on November 5, 1976. The cells were grown in Leibovitz L-15 medium, supplemented with inactivated fetal bovine (15%) serum and tryptose phosphate broth (10%). At their arrival, the cells had been subcultured 183 times. They are now in their fourth transfer at YARU. Split ratios are usually 1:2, occasionally 1:3. Storage in liquid nitrogen will be attempted as soon as feasible. Thereafter, infection experiments are planned.

Comparison of efficacy of Freund's adjuvant and sarcoma-180 in mice for preparing ascitic fluids (G. Roze and W. Downs) Titers of CF antibody in mouse ascitic fluids were compared after 4 different immunization schedules with yellow fever virus. Two parameters were varied 1) purity of the virus inoculum (mouse brain) and 2) method of induction of ascites, Freund's complete adjuvant (FCA) versus FCA plus sarcoms-180 T/G. Weanling mice were inoculated ip with 10% mouse brain containing 6.9 log LD_{50} per injection. The schedule of inoculations was:

Day	
0	FCA + virus
7	FCA + virus
14	FCA + virus
16	FCA
21	FCA + virus

There were 4 groups of 10 mice:

Group 1 virus centrifuged 1500 rpm, 15 min, no sarcoma 180
Group 2 virus centrifuged 10000 rpm, 1 hr, no sarcoma 180
Group 3 virus centrifuged 1500 rpm, 15 min, sarcoma day 21
Group 4 virus centrifuged 10000 rpm, 1 hr, sarcoma day 21

Paracenteses were done between the days 4 and 25 after completion of immunization. The CF titers on the 21st day after completion of immunization were representative:

Schedule	CF titer day 21	Total volume all tappings
1	128	76 ml
2	64	37 ml
3	64	469 ml
4	16	186 ml

It was concluded that while sarcoma-180 yielded larger volumes, the absolute amount of antibody was not increased over FCA alone. In this experiment there was very little anticomplementary activity in any of the schedules. Purification of the immunizing antigen by centrifugation appeared to result in a lower antibody response.

RNA-RNA homology studies with the VEE virus complex (C. Frazier with A. Schluederberg) This work is part of an ongoing project designed to examine the genetic homologies among strains in order to explore further some aspects of strain variation and the epidemiology of the VEE complex.

During the past year two new techniques have been explored and have resulted in the repeated successful isolation of the complementary strand of RNA needed for the hybridization procedure.

Dale and Ward (Biochemistry 14: 2458-69, 1975) described a procedure for selecting specific RNAs from a mixture of RNAs, DNA, and protein based on the selective binding of mercurated species to sulfhydryl agarose. Mercury can be covalently bound to selected RNA species without seriosly altering the annealing properties of the RNA. The mercurinucleotides thus formed have a high affinity for the sulfhydryl groups on the resin, and therefore are retained on the resin while the non-mercurated species are washed through with buffer. The mercurinucleotides can then be recovered from the resin by eluting with buffer that contains a competing mercaptan, such as mercaptoethanol. In an attempt to obtain complementary stranded RNA from the replicative forms (RF) and relicative intermediates (RI) present in VEE infected cells, the following procedure was employed. Single stranded RNA was isolated from purified virions and mercurated. The mercurinucleotide product was then used as a probe to isolate the complimentary strand from a cytoplasmic extract prepared from infected cells by annealing the probe with the heat denatured components of the extract. The probe--complimentary strand complex--was then separated from the other species by means of column chromatography on sulfhydryl agarose. The initial results were unsatisfactory so two modifications were made in the technique.

The problem of P^{32} labeled species other than the mercurated RNA binding to the resin was surmounted by treating the cytoplasmic extract with SDS:phenol:chloroform:octanol before the denaturation process. Better results were achieved if DMSO replaced heat as the denaturation agent.

The major breakthrough was the inclusion of 1, 5-Naph-thalenedi-sulfonic acid Disodium Salt (NDSA) in several procedures as an RNAse inhibitor. Not only was complimentary strand isolated repeatedly and consistently with the sulfhydryl agarose system, but double stranded RNA (Beck-Wycoff strain) was also isolated via the Franklin column. Numerous attempts to isolate dsRNA from preparations from cells infected with various strains of VEE using the Franklin column had failed in the past, despite the fact that dsRNA from Reo virus could be successfully separated on the column. DsRNA could also be obtained from Sindbis infected cells using the Franklin column without the presence of NDSA. Experiments indicated that the failure to isolate routinely dsRNA from cells infected with VEE was due to degradation by RNAse. NDSA, in all procedures to date, has significantly reduced this degradation, without creating adverse side effects such as dissolving centrifuge tubes and disrupting column beds, which are noted with some inhibitors.

The procedure using mercurated probes and sulfhydryl agarose (SHA) column chromatography was chosen over methods employing the Franklin column for several reasons. With the SHA system all of the label is in the complimentary strand so it is not necessary to assume that the label is evenly divided among the two strands in determining when saturation has occurred during annealing experiments. This system also presents an easy method (column chromatography on SHA) to remove any dsRNA from the system that is a product of self-annealing or non-denaturation and not hybridization. Most importantly the chromatography on SHA is a faster procedure than chromatography on a Franklin column. Since a higher percentage of counts is recovered as complimentary RNA with the SHA technique, (Table 33) it is postulated that the RNA is exposed to less degradation due to the shorter chromatography time. In addition, the "dsRNA" obtained from the Franklin column was substantially susceptable to RNAse which indicates that there is a considerable amount of ssRNA in the preparation. Salting out of the RI's did not yield a pure ds sample (Table 33).

Although, sufficient labeled material could be obtained by this procedure (approcimately 4 ml of about 1×10^6 cpm/ml per experiment), there have been difficulties in obtaining saturation in annealing experiments. The material is RNA since treatment with DMSO and RNAse renders it acid soluble. It is not degraded during the annealing procedure since non-digested controls remain acid precipitable. Increasing amounts of the complementary strand are annealed when increasing amounts of virion RNA are added, but saturation has yet to be reached. It is possible that the material has a low specific activity.

Table 33

Comparison of "dsRNA" isolated from Franklin and SHA columns

RNA	Column	Virus	Label	% dsRNA[1]	% RNAse Resistant[2]	% Complimentary[3] strand
Total	Franklin	VEE (BW)	P^{32}	0.44	41.18	0.09
Salt soluble	Franklin	VEE (BW)	P^{32}	0.61	28.53	NA
Salt insoluble	Franklin	VEE (BW)	P^{32}	28.76	45.90	NA
Total	Franklin	Sindbis	H^3	15.70	41.70	3.27
Total	SHA	VEE (BW)	P^{32}	NA[4]	NA	8.00

[1] $\frac{\text{counts in ds region}}{\text{total counts eluted}}$

[2] $\frac{\text{RNAse resistant counts}}{\text{total counts in sample}}$

[3] $\frac{\text{fraction counts in ds region} \times \% \text{ RNAse resistant}}{2}$

[4] not applicable

This has not been measured directly. Double label experiments, using H^3 labeled virion RNA and P^{32} labeled complimentary strand RNA are underway to determine if saturation in annealing can be achieved by adjusting the amount of virion RNA and/or the volume of the annealing reaction mixture.

The fluorescent focus assay with arboviruses in vertebrate and in Aedes albopictus cells (G. Tignor, J-P. Digoutte, A. Smith, D. Knudson, and S. Buckley). Since fluorescent focus formation has proved useful with rabies virus in making primary virus isolations in CER cells and in enumerative assays (Smith, A.L., Tignor, G.H., Mifune, K., Motohashi, T. Isolation and assay of rabies serogroup viruses in CER cells. Intervirology, in press), studies have been undertaken to compare the relative sensitivity of a plaque assay under solid overlay with that of the fluorescent focus method in enumerative assays for arboviruses. Antibody titers have been compared when determined by fluorescent focus inhibition and plaque reduction tests. The results demonstrate that the fluorescent focus test is as sensitive as plaque formation and much more rapid in enumerative assays (Table 34). These features become especially convenient in assaying viruses with long incubation periods. Antibody titers (50% reduction) as determined by the two methods are identical (Table 35) (Tignor, G.H. et Digoutte, J-P. Etude comparative de deux alphavirus par la methode des plages et par comptage des foyers fluorescent en cellule vero. Ann. Micro. (in press).

Fluorescent focus formation has also been used to assay infection of Aedes albopictus cells by Sindbis virus which does not cause cytopathic effect in these cells. Infective titers have been determined in A. albopictus cells grown at both room temperature and in cells grown at 37°C (Table 34) (Digoutte, J-P., Tignor, G.H., Smith, A.L., and Knudson, D.L. Comptage rapide de l'infectivite du virus Sindbis en lignee cellulaire d'Aedes albopictus par numeration de foyers fluorescente. Submitted to Ann. Micro. [1976]).

Growth of arboviruses in CER cells (A. Smith and G. Tignor). CER cells were developed in Japan and shown to be efficient for isolation of rabies viruses (Smith et al., Isolation and assay of rabies serogroup viruses in CER cells, Intervirology, in press, 1977). They have also proved efficient for growth of a wide variety of arboviruses. Representative data of infectivity in mice and CER cells of EEE, WEE, Flanders, SLE, and CE viruses are shown in Table 36. For PFU and fluorescent focus unit (FFU) determinations a sephadex overlay was used.

Three strains of dengue virus grew in CER cells. Two-chamber Lab-Tek slides were seeded with 10^6 CER cells per chamber and held in 5% CO_2 for 24 hours at which time they were inoculated with 3.8, 3.1 and 4.1 log

Table 34

A comparison of fluorescent focus formation (FFU) and plaque formation (PFU) as methods for enumeration of virus particles

Virus	Host Cell	Method	Time of reading after infection	Titer per ml
CHIK	VERO	FFU	17 hrs	3×10^8
		FFU	2 days	6×10^8
CHIK	VERO	PFU	4 days	2.5×10^7
		PFU	8 days	1.7×10^9
Sindbis	VERO	FFU	30 hrs	2.4×10^7
Sindbis	VERO	PFU	4 days	6.3×10^7
		PFU	8 days	7.5×10^7
Dengue 2	LLCMK2	FFU	3 days	3.6×10^7
Dengue 2	LLCMK2	PFU	8 days	3.2×10^7
Semliki	VERO	FFU	30 hrs	1.7×10^8
Semliki	VERO	PFU	8 days	1.5×10^9
Sindbis	Aedes albopictus room temperature	FFU	17 hrs	3.6×10^6
			41 hrs	1.4×10^7
Sindbis	Aedes albopictus 37°C	FFU	24 hrs	3.1×10^7
			48 hrs	3.6×10^7
Murutucu	VERO	PFU	12 days	5.8×10^5
Murutucu	VERO	FFU	3 days	2.0×10^6
Oriboca	VERO	PFU	12 days	2.2×10^5
Oriboca	VERO	FFU	3 days	2.7×10^5

Table 35

Titer of Chikungunya and Sindbis immune sera in homologous tests as determined by plaque reduction (PRNT) and fluorescent focus inhibition (FFI)

	Virus			
	Chikungunya		Sindbis	
Antibody	PRNT	FFI	PRNT	FFI
Normal serum	10		10	10
Chikungunya	80*	80		
Sindbis			40	80

*Inverse of the dilution which gives 50% plaque reduction. Virus dose was 1.5 log PFU or FFU.

Table 36

Infectivity assays of 5 arboviruses in CER cells

Virus	Strain	Mouse Passage	SMICLD$_{50}$*	TCID$_{50}$	PFU	FFU
EEE	Sp 687	2	$\geq 10^{10}$	10^{10}	$10^{9.4}$ (36 hr.)	
WEE	Sp 72666	3	$10^{8.0}$	$10^{7.0}$	$10^{7,4}$ (36 hr.)	
Flanders	Prototype	3			$10^{4.0}$ (day 4)	$10^{3.4}$ (day 3)
SLE	Parton	16		$10^{6.0}$	$10^{6.8}$ (day 4)	$10^{6.9}$ (day 2)
California	BFS 283	23		$10^{4.0}$	$10^{4.5}$ (day 2)	
					$10^{4.7}$ (day 3)	

* Suckling mouse ic LD_{50}

** Titers are per ml.

mouse ic LD_{50} respectively of either Dengue 1 (Hawaii), Dengue 2 (NGC), or Dengue 4 (H241) or with phosphate buffered saline. After 1 hour of adsorption at 37°C, the inoculum was washed off the cells, and MEM-E containing 2% fetal calf serum was added. The cultures were maintained at 37°C in 5% CO_2. They were observed daily for cytopathic effect. Fluid and cells were harvested on days 1, 2, 3 and 5 for titration of released virus in CER cells under Sephadex overlay, and fluorescent antibody staining, respectively. The results are shown in Table 37. The fluorescence of cells in fluid cultures was highly focal indicating virus spread from cell to cell.

Growth of arboviruses in cells of neuronal origin (G. Tignor) The growth of mouse neurovirulent and avirulent strains of selected arboviruses is being examined using neuroblastoma cells and selected cells of non-neuronal origin. In the case of yellow fever and SLE, neuroblastoma cells (clone N18) have been found to be non-permissive to mouse-brain derived viscerotropic strains and permissive to neurotropic strains (Table 38). In the case of Sindbis virus, the mouse neurovirulent strain (Sa Ar 86) multiplies to a lower titer in non-neuronal cells (CER, L929 and rat glioma) than does the avirulent strain (Table 39). The mechanism controlling permissiveness in neuronal and fibroblast cells is under study beginning with the first step in the replicative cycle, attachment.

Mokola complement-fixing antigen in invertebrate cells (S. Buckley) Specific complement-fixing antigen has been detected in Mokola virus persistently-infected Aedes albopictus cells. Carrier cultures, established in February 1974 with a cloned virus preparation, have since been transferred over fifty times. The combined cell-culture fluid was used as the antigen source without further manipulation such as freezing and thawing cycles or clarification by centrifugation. Complement-fixing titers varied from 1:4 to 1:16. For reasons of economy, the complement fixation test will be used in the future for monitoring presence of Mokola virus in the carrier cultures and will replace the more expensive methods such as plaque assay in Vero cells or intracerebral inoculation of newborn mice.

Serologic surveys

Serologic survey of West Africa (J. Casals) In association with Dr. J.D. Frame, College of Physicians and Surgeons, Columbia University, New York, a serological survey is being conducted in certain African countries. The main purpose of the survey is to determine current arbovirus and Lassa virus infections in a population which, by its

Table 37

Growth of 3 strains of Dengue virus in CER cells

		Day 1	Day 2	Day 3	Day 5
	CPE	0	0	0	1+
Dengue 1	% FA	<<1%	<<1%	1%	10%
	*FFU/ml	$<10^{0.95}$	$10^{0.95}$	$<10^{0.95}$	$10^{2.95}$
	CPE	0	0	1+	2+
Dengue 2	% FA	1%	5-10%	60%	90%
	FFU/ml	$10^{2.3}$	$10^{2.95}$	$10^{3.6}$	$10^{3.7}$
	CPE	0	0	0-1+	2+
Dengue 4	% FA	5%	40-50%	50%	50%
	FFU/ml	$10^{2.0}$	$10^{3.4}$	$10^{1.95}$	$<10^{0.95}$

*Fluorescent Focus Units

Table 38

Virus infection in selected cell lines and mice

Virus Strain	Cell Line				Mice (i.c.)	
	CER		N 18		Infant	Adult
	susceptibility	CPE	susceptibility	CPE		
SLE (Parton) p 16	high*	+	high	+	+	+
SLE (BeAr 23379) p 2	high	+	low**	-	+	-
SLE (BeH 203235)	high	+	high	+	+	+
Yellow Fever, French viscerotropic p 1	high	+	low	-	+	+
Yellow Fever, French viscerotropic, high pass	high	+	high	+	+	+
Yellow Fever (17D vaccine)	high	+	low	-	ND	ND
Rabies (CVS) many	high	+	high	+	+	+
VEE (Beck Wycoff) p 7 VERO	high	+	high	+	+	+
VEE (TC 80) p 1, VERO 1	high	+	low	-	+	-

*high: 100% of cells can be infected after 2-3 days of incubation at MOI from 1.0 to .001

**low: fewer than 1% of cells can be infected after 2-3 days of incubation even if the cells were infected at a MOI of more than 1.0 PFU/cell

Table 39

Comparison of prototype strain of Sindbis virus with a South African strain pathogenic for adult mice: fluorescent focus assays in different tissue culture systems

	*FFU/ml			$ICLD_{50}$
Virus	CER	C6[a]	L929[b]	Adult Mice
EgAr 339 (prototype) P. 14	9.9×10^7	1.2×10^8	3.15×10^6 (foci)	$<9.0 \times 10^2$
SaAr 86 p. 4	7.2×10^4	1.6×10^5	2.5×10^4 (single cells)	7.8×10^6

*Fluorescent Focus Units

[a]rat glioma cells

[b]mouse connective tissue cells

nature, is readily and repeatedly accessible. The population consists of staff members of Missionary hospitals and hospital patients that present with fevers of unknown origin (FUO), and missionaries and their families that are seen for medical check-up on their return to U.S. on leave every two years. The hospitals participating in the survey are for the most part in Nigeria, Liberia, and Cameroon.

Since the purpose of the survey is to diagnose and monitor recent infections, the complement-fixation (CF) test was chosen for the purpose, as it is generally more indicative of recent infections than other serological tests. The antigens used are for arboviruses that previous experience in this laboratory had shown to be prevalent in West Africa, as follows: chikungunya, Sindbis, yellow fever, Dengue type 2, West Nile, Zika, Wesselsbron, Bwamba, and Bunyamwera; in addition antigens for Congo-Crimean hemorrhagic (CCHF) fever and Lassa fever were included.

The total number of sera tested to date is 716; this includes tests done in 1975 and 1976, in approximately equal numbers each year; the combined results are given in Table 40. Since this material is still under study no effort has been made to show in the table the separate and various groups: medical personnel, patients, missionaries.

The results in the table show the high prevalence of infections with chikungunya (or o'nyong-nyong-ONN) during the period covered, 1975-76, in the population surveyed. A number of secondary immune responses for group B viruses were undoubtedly due to vaccination against yellow fever, followed by infection by another virus of the group, probably Zika, or Wesselsbron, or some other virus not included in the survey. Worthy of note is the fact that several reactors with Lassa antigen were uncovered; and of particular interest is the fact that a serum reacted with CCHF antigen, this being the only one so far encountered in the survey.

Serologic survey of Ghana (N. Quao and W. Downs). A serologic survey for HI antibody to chikungunya, Sindbis, Dengue 1, Uganda S, Wesselsbron, West Nile, yellow fever, Zika, Bunyamwera, and Ilesha viruses was conducted for 216 Ghanaian sera from persons between 20 and 29 years of age. These sera represented residents of 8 different regions of the country. Table 41 shows the results analyzed according to whether the reaction was specific (4-fold or greater titer than to others in the same group), superinfection (titer of at least 1:40 and not more than 2-fold difference between 2 viruses of the same group), or undiagnosable (titer not over 1:20). Pertinent findings were: a) One hundred and fifty-four (71%) of the sera tested were positive for the alphaviruses. There were 113 specific reactors of which 112 were to chikungunya and one to Sindbis. b) One hundred and twenty-four (57%) of the 216 sera were positive to the flaviviruses. Eight of those positive gave specific reaction with Zika, three with yellow fever and two each with West Nile and Wesselsbron and one with Dengue 1.

Table 40

Complement-fixing antibodies for selected virus antigens in sera from missionaries, and medical staffs and patients with undiagnosed short febrile illnesses in hospitals in Africa, mainly Nigeria, Liberia and Cameroon.

Serum specimens, number	Number unusable*	Diagnostic	Negative**	Number of sera positive for antigen												
				CHI	SIN	YF	D2	ZIK	WN	WES	GB	BUN	BWA	CON	LF	?
716	80	636	422	108	2	22	24	10	8	3	27	11	6	1	14	11

*Non-specific or anticomplementary.

**First dilution of serum, 1:4, except for Lassa antigen where undiluted serum was employed.

Antigens used: chikungunya, Sindbis, yellow fever, dengue 2, West Nile, Wesselsbron, Bunyamwera, Bwamba, Congo-Crimean hemorrhagic fever and Lassa fever.

GB indicates that the serum had a group B superinfection-type response.

?, no diagnosis could be made.

Table 41

Results of arbovirus hemagglutination-inhibition tests on 216 Ghanaian men and women
Ages 20-29

Reaction	Group	Total Positive	Type of Reaction		Virus probably responsible for antibodies (specific)	
Positive, 176* any virus	Alphavirus	154	Specific	113	Chikungunya	112
			Superinfection	33	Sindbis	1
			Undiagnosable	8		
	Flavivirus	124	Specific	16	Dengue 1	1
					Wesselsbron	2
					West Nile	2
					Yellow Fever	3
			Superinfection	94	Zika	8
			Undiagnosable	14		
	Bunyavirus	47	Specific	10	Bunyamwera	2
					Ilesha	8
			Superinfection	9		
			Undiagnosable	28		
Negative, 40 all viruses						

*Alpha-and Flaviviruses were involved together in 90 positives; Alpha-and Bunyaviruses in 10 positives; Flavi-and Bunyaviruses in 6 positives; Alpha-Flavi-and Bunyaviruses in 21 positives.

reagents for CHF-Congo, yellow fever, and group B hemorrhagic fever viruses to WHO for use in Pakistan. In addition, CHF-Congo antigen and a battery of antigens and sera for a wide variety of viruses known in India, other parts of Asia, and Africa were supplied to CDC for diagnostic study of the outbreak.

Dr. P.A. Webb of the Special Pathogens Branch, CDC found one of the convalescent sera to be positive in the 1:64 dilution with the CHF-Congo CF antigen. Five sera from 3 patients were submitted as acetone extracted specimens to YARU for HI tests. Results are shown in Table 42. Identical HI results were obtained using goose cells and using trypsinized human O cells. All sera from Pakistan were positive for CHF-Congo antigen; all human control sera were negative. The Pakistan sera did not react with the related virus, Hazara. On the basis of the HI tests it was possible to make a presumptive diagnosis of Crimean hemorrhagic fever.

Table 42

Diagnosis of Crimean hemorrhagic fever in Pakistan outbreak in 1976. HI results

Sera	Date	Titer (4 antigen units)	
		Congo-CHF	Hazara
Dr. S	12 Feb	80	0
Dr. S	18 Feb	80	0
Dr. ZK	21 Feb	20	0
Dr. ZK	24 Feb	20	0
Dr. W	21 Feb	>160	0
Congo mouse (Positive control)		>80	20
AR (negative control)		0	0
CA (negative control)		0	0
JVL (negative control)		0	0

c) Forty-seven (22%) of the 216 sera were positive to the bunyaviruses and of these eight were specific reactions to Ilesha and two to Bunyawera.

There were no clear-cut differences when data were analyzed by population size of the town of residence. For alphaviruses and flaviviruses, the percentage of positives was higher in zones of rainfall <50 inches, and higher in the savannah woodland zone than in the high forest. The bunyavirus reactors were more common in the high forest zone.

Serologic survey of Quebec (R. Shope). Twenty human sera from residents of Quebec Province, Canada were referred by Dr. H. Artsob, University of Toronto, for confirmation of a high rate of HI positivity to SLE antigen. All sera were negative for SLE HI reactions at YARU.

Yellow fever immunity of YARU staff (W.Downs, G. Roze, and H. Siebens). Staff and students are vaccinated for yellow fever before beginning to work at YARU. Tests for N antibody were previously done in mice. In 1976, the 17D yellow fever plaque reduction neutralization test was used in VERO cells. Fifty-four sera of vaccinated individuals were examined at the 1:8 dilution in 4 tests. Plaque counts in non-immune serum were 54, 68, 79, and 62. Counts in vaccinees were:

Plaque number	Number of sera
≤10	40
11-20	8
21-30	4
31-40	1
>40	1

The 2 sera which had less than 50% reduction were from persons vaccinated more than 10 years ago. This test appears to be suitable for yellow fever sero-immunity surveys.

Diagnosis of disease

Outbreak of Crimean hemorrhagic fever in Pakistan (J. Casals and D. Clarke) In January and February 1976, cases of viral hemorrhagic fever were reported from Pakistan. The National Health Laboratories at Islamabad submitted through WHO channels, 13 serum specimens from 9 cases to CDC, Atlanta, Georgia. Two of the cases had paired specimens, and from one case a convalescent serum was available. YARU supplied

Testing of cases of arthrogryosis and hydranencephaly with Simbu group viruses (R. Shope). Arthrogryposis and hydranencephaly (ACH) of cattle is caused in Japan, Australia, and Israel by Akabane virus of the Simbu group. The disease occurs in Western USA and Canada and is a significant cause of fetal wastage. Sera of cattle from Saskatchewan were supplied by Dr. J.R. Saunders of the University of Saskatchewan. Sixteen sera from cases of ACH were tested at 1:4 by CF and at 1:10 by HI with Buttonwillow and Mermet (CF and HI) and with Manzanilla (CF only). An additional 84 sera, some of them survey sera, were tested by HI with Buttonwillow and Mermet. All tests were negative. Neutralization tests with Akabane were carried out at Plum Island by Dr. C. Campbell and were also negative.

Testing of cases of Alzheimer's disease with orbiviruses (R. Shope) Sixty-two sera of Alzheimer's disease patients were supplied by Dr. B. Henderson, UCLA. The sera were tested by CF for antibody to reovirus 3 and 17 orbiviruses. Five sera reacted non-specifically with mouse brain. There were 2 sera each reacting at 1:4 (only) with Wad Medani with D'Aguilar antigens. All other tests were negative, except for some expected reactions with reovirus-3.

Virus isolation attempts from suspect legionnaire's disease and dengue (S. Buckley) Case #1: Raymond Brunner. Diagnosis: Fever of unknown origin, query legionnaire's disease. Specimens (serum, urine, cerebrospinal fluid, stool, throat) were submitted to YARU by Earl B. Byrne, M.D., Infectious Disease, Brywn Mawr Medical Specialists Association, Bryn Mawr, Pennsylvania 19010, on 8/7/76. Specimens were inoculated into VERO and BHK-21 cell cultures following adequate processing. The cultures were observed for development of a cytopathic effect (CPE) for the period of 21 days. In addition, three blind passages were carried out in BHI-21 cell cultures with the serum specimen collected on 8/5/76. No cytopathic agent was isolated.

Case #2: Sidney Smith. Diagnosis: Dengue. Serum collected on 5/26/76 was submitted by Dr. Vincent T. Andreole, Professor Medicine, Yale University. The specimen was accompanied by a note indicating that Sidney Smith, a graduate of the Department of Anthropology, had returned from the Philippines with a fever of unknown origin. The tentative diagnosis was dengue. The serum specimen was inoculated into LLC-MK_2 cell cultures (a) under fluid; (b) under agar, and also into Aedes albopictus cell cultures (under fluid). The vertebrate cell cultures were incubated at 37°C and the invertebrate both at 30°C and 37°C. All cultures were observed for development of CPE, especially syncytia formation. In addition, fluids of the Aedes albopictus cell cultures were subinoculated at weekly intervals into LLC-MK_2 cell cultures maintained under agar. No cytopathic agent was isolated.

Search for a viral etiology of Lyme arthritis (R. Shope, in collaboration with Dr. A. Steere and S. Malawista, Department of Medicine) Steere, Malawista, Snydman, Shope, Andiman, Ross, and Steele have described arthritis and rash in patients from Lyme, Connecticut and 2 nearly rural villages. From June 1972 through March 1976, 48 residents (36 children and 12 adults) of the three communities developed an apparently similar type of arthritis. The illness was characterized by the sudden onset of asymmetric pain and swelling in 1-4 large joints, usually the knee, lasting a median of one week (range .3-24). Thirty-five patients (78%) had recurrent attacks (median, 3; range 1-10) lasting a median of one week (range .15-16) with a median of 2.4 months (range .25-23) between attacks. Thirteen patients (27%) had an erythematous plaque which expanded forming a raised, indurated red ring (erythema chronicum migrans Afzelius) a median of 4 weeks (range 1-24) before the onset of arthritis. The overall attack rate was 4 cases per 1,000 residents, but the attack rate among children living on 4 roads was 1 in 10; 5 families had more than one affected member. Thirty-three patients (69%) had the onset of symptoms from June through September. No patient had iridocyclitis or a positive test for antinuclear antibodies through 8 of 18 patients symptomatic at the time of the study had low serum C_3 levels (median 61; range 48-70mg%). Cryoglobulinemia was a prominent finding. Cultures of synovium and synovial fluid and serologies for agents known to cause arthritis were not suggestive of bacterial or mycoplasma infection. Although the articular symptoms of many patients were compatible with oligoarticular juvenile rheumatoid arthritis, it is probable that Lyme arthritis is a previously unrecognized entity, the epidemiology of which suggests that it may be an insect-transmitted illness.

Previous serologic testing with arboviruses yielded negative results. Sera of 5 patients with acute onset during the summer of 1976 were tested by CF 3 weeks to 2 months after onset. They were negative to 216 viral antigens (Table 9). In addition, the same sera plus a purified, redissolved cryoglobulin, when tested by CF with Powassan and arenavirus antigens, were all negative. Acute phase sera from 2 patients with fever and rash (prior to arthritis) were inoculated i.c. in baby mice. No pathogenic agent was isolated. The etiology of the disease remains unknown.

Field studies in Connecticut

Entomologic study of Lyme arthritis (R. Wallis, T. Aitken, A. Main, and K. Kloter) The objective of this study is to find the possible arthropod vector transmitting Lyme arthritis. Initially the approach was by analysis of biting arthropods collected in the epidemic area as compared with those collected in a control area where cases are not known to occur.

For the past 13 years the Section of Medical Entomology and YARU have conducted field work for surveillance of eastern equine encephalitis in Connecticut, and from 1974, sponsored in part by the Connecticut State Department of Health, these studies have been concentrated in the upper Hammonassett River Basin. This area is 20 miles west of the Lyme, Old Lyme, and East Haddam (east of the Connecticut River) area where the concentration of Lyme arthritis cases occur, and was considered a suitable control area for comparison of arthropod collections from the epidemic area (In epidemiologic studies only three cases were identified west of the Connecticut River even though physicians were contacted in communities as far west as the Hammonassett River. All three of these cases may have exposures in the Lyme area).

Therefore during the summer of 1976, beginning in May, arthropod collection was initiated in the two areas. Light traps were operated five nights each week, and baited small mammal traps were set to catch small wild mammals for ectoparasite examinations.

From May through September, 1976, approximately 104,600 biting arthropods (mosquitoes, black flies, sand flies, horse and deer flies, plus 774 ticks) were collected in the pilot field study. They were sorted, identified, pooled by species and day of collection, and processed for virus isolation tests. The 77 species of biting arthropods collected, included: Mosquitoes (Culicidae) - 23 species; Black flies (Simuliidae) - 11 species; Snipe flies (Rhagionidae) - 3 species; Sandflies (Ceratopogonidae) - 12 species; Horse and Deer flies (Tabanidae) - 24 species; Phlebotomine sandflies (Psychodidae) - 1 specie; and Ticks (Ixodidae) - 2 species. Most of the pools of these species have been tested by inoculation into infant mice for virus isolation and results shown in Table 43.

In comparing the composition of the biting arthropod population in the Lyme area with that in the Hammonassett control area a number of differences were found. The Lyme area collection contained a number of species that did not occur in the control collections. These included an additional species of mosquito (Orthopodomyia signifera), 5 additional species of black flies (Cnephia muttuto, Prosimulium fuscum, P. magnum, and Simulium jenningsi), 6 additional species of sand flies (Culicoides guttatus, C. obsoletus, C. travisi, C. piliferus, C. bigttatus, C. haematopotus), 3 additional species of horse flies (Tabanus trimaculatus, T. marginalis, T. lineola), 4 species of deer flies (Chrysops sackeni, C. obsoletus, C. niger), and four specimens of one species of Phlebotomine sandfly (Brumptomyia vexator).

Finding this species of Phlebotomine sandfly by Dr. Aitken in East Haddam, Connecticut, is of particular interest because species of this medically important group have not been reported previously in the Northeastern United States. Also, of particular interest in comparison of the collections from the two areas east and west of the Connecticut River,

Table 43

Possible virus isolations* from female arthropods collected in Connecticut during 1976

Strain	Species	Number	Date Collected	Area
Ar-64-76	Aedes abseratus	33	2-3 Jun	Hammonasset River (Site I)
Ar-443-76	Coquillettidia perturbans	94	6-7 Jul	Clinton (Aquarius Stables)
Ar-473-76	Culex restuans	25	23 Jun-30 Jul	New Haven (Morris Cove)
Ar-505-76	Coquillettidia perturbans	100	1-2 Jul	Hammonasset River (Site I)
Ar-831-76	Culiseta melanura	33	26-27 Aug	Hammonasset River (Site II)
Ar-856-76	Aedes aurifer	23	1-15 Jul	Hammonasset River (Sites I & II)
Ar-881-76	Culex restuans	35	8-9 Aug	Clinton (Aquarius Stables)
Ar-887-76	Culex restuans	43	24-26 Aug	Clinton (Aquarius Stables)
Ar-986-76	Culex restuans	32	20-30 Sept	East Haddam
Ar-1050-76	Culiseta melanura	21	1-28 Sept	Lyme
Ar-1086-76	Culex restuans	39	14-15 Sept	Clinton (Aquarius Stables)

*Ar-64-76 is a California group virus; all others are unidentified

was the distribution and number of ticks of the species Ixodes scapularis on the small mammals that were trapped. In the Lyme arthritis epidemic area, 472 I. scapularis were collected from approximately 250 rodents, as compared to only 8 I. scapularis found on 125 mice trapped inthe Hammonassett River control site, indicating approximately 30 times as many of these ticks in the epidemic area. It is known that the preferred hosts of the larvae and nymphs of this tick are small rodents and that the hosts of the adult are primarily deer.

This led to the hypothesis that the occurrence of Lyme arthritis only in recent years (1972-76) and the nidality of the cases in the epidemic area were associated with the distribution and abundance of the deer tick, I. scapularis and the number and distribution of its preferred host--the deer in Connecticut. Since it is known that the tick population in New England occurs predominently in the southerly coastal area where there is a more moderate climate than in the northern upland area, the history and distribution of the deer population in Southern Connecticut was examined in data provided by Dr. Paul Herig, Chief of the Deer Biology Section of the State Environmental Protection Agency. A number of facts were found that support the hypothesis stated above. The deer population in Connecticut has been increasing gradually during the past 100 years due to protection of the deer by strict laws controlling hunting. From an estimated less than a dozen in the middle of the 19th century, there are now an estimated 3,000 in 1976. While the total number has increased in the state, in the coastal areas the distribution is uneven. The State Deer Biology Section initiated aerial photographic sampling of the deer population in 1974 and anlaysis of their data for 1974 and 1975 indicates a three fold build up of the population in the Lyme arthritis study area east of the Connecticut River in the last two years to an estimated density of 7.5 per square mile. In comparison, on the west side of the river, including the Hammonassett study area, there were only two sightings in 1974 and none in 1975, or an estimated density of 1 per square mile.

Currently, field work is in progress to sample empirically the distribution of deer and of the deer tick, I. scapularis in relation to the clustering of cases of Lyme arthritis. During the hunting season from November 22 to December 22, hunters are allowed to bag deer only by permit and must register their specimens at state deer weighing stations located throughout the state. As freshly killed deer are brought into these statons, we are obtaining blood samples and collections of ticks from them. The final results of this effort are not yet available, but initial sampling indicates that in the southern coastal area of Connecituct most of the deer are east of the Connecticut River with a particular concentration of them in the Lyme arthritis epidemic area, and few west of the river. Deer from northern upland areas are generally lacking I. scapularis whereas those in the coastal areas particularly in the Lyme region are parasitized by ticks.

Distribution of reagents, WHO Collaborating Centre for Reference and Research.

Distribution of reagents, World Health Organization-Collaborating Centre for Reference and Research (R. Shope, J. Casals, and S. Buckley) The equivalent of 615 ampoules of arbovirus reagents were distributed from the WHO Centre to laboratories in 23 countries during the period January 1, 1976 to December 31, 1976. This total consisted of 349 ampoules of virus stock, 104 ampoules of virus antigen, and 162 ampoules of mouse ascitic fluid or immune sera.

Of the virus stocks distributed, this represented 265 different arboviruses; of antigens, 40 different arboviruses; and of sera, 69 different arboviruses (not counting individual viruses represented in polyvalent ascitic fluids).

During this same period, the equivalent of 861 ampoules of arbovirus reagents were referred to this Centre from laboratories in 20 different countries. The referrals consisted of 371 ampoules of virus specimens (Table 44), 209 ampoules of virus antigens, and 281 ampoules of immune reagents. In addition, 599 sera were received for arbovirus antibody survey testing.

Six different cell lines were distributed during 1976: 12 Aedes albopictus, 6 Aedes aegypti, 2 BHK-21, 2 Vero, 9 CER, and 2 LLC-MK_2. The recipients are listed in Table 45. The continuous mosquito cell lines are grown and maintained in Mitsuhashi-Maramorosch medium supplemented with 20% inactivated fetal bovine serum.

Table 44

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Australia			
CSIRO-19	Culicoides	new to region, ungrouped possible orbi virus	
CSIRO-25	Culicoides	new to region, ungrouped	
MRM 14556 (Ngaingan)	Culicoides	new to region, ungrouped	
MI 19334	Ixodes uriae	new to region, ungrouped	
Brazil			
H 34675	man	group B, Sao Paulo encephalitis	Group B, new
Be An 213452 (Itaituba)	Didelphis	Phlebotomus fever group; Candiru-related	new virus, phlebotomus fever group
Be An 157575	Pyriglena	related to Kwatta	Kwatta subtype
Be Ar 177325 (Inhangapi)	Lutzomyia	ungrouped	ungrouped, presumably new
Be An 174214 (Araguari)	Philander	ungrouped	ungrouped, presumably new
Be An 238758 (Santarem)	Oryzomys	ungrouped	new; CF cross reaction with Congo
Be An 293022	Oryzomys	Tacaribe group	Tacaribe group, new to Brazil
Be Ar 202527	Culex portesi	Aruac-related	Aruac virus
Be An 235467	Agouti	Triniti-related	Triniti virus
Be An 109303 (Ananindeua)	Caluromys	Group Guama	Group Guama, new
Be An 116382 (Timboteua)	sentinel mice	Group Guama	Group Guama, new
Be An 228950	frog	ungrouped	viable, but low titer
Be An 153564 (Benevides)	sentinel mice	Capim group	new member of Capim group

Table 44 cont.

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Bulgaria			
Cz 335/336	Haemaphysalis		Bhanja
Cz 326	Haemaphysalis		Bhanja
Egypt			
Eg Ar T 4137	Argas	Hughes group	
Ethiopia			
Ar 2316	Culicoides	Simbu gruop	
Ar 2777	Amblyomma	Dugbe	
Ar 3056	Amblyomma	Dugbe	
Ar 3102	Amblyomma	low titer, ungrouped	
Ar 3201	Culicoides	related to Ar 662	
An 3307	Thrush	Group B	
Ar 3554	Rhipicephalus	Different from Ar 3559, DCA resistant	
Ar 1618	Mansonia	ungrouped, DCA resistant, related to Ar 662 and Ar 1180	
An 3530	Boubou	ungrouped, DCA sensitive	
Ar 3559	Rhipicephalus	Different from Ar 3554, DCA sensitive	
An 3662	Thrush	Group B	
An 3785	Puff back	Dugbe	
An 4152	Thrush	Group B	

Table 44 cont.

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Ethiopia (cont.)			
Ar 838	Hyalomma	Congo	
An 808	Arvicanthis	phlebotomus fever group	
An 798	Arvicanthis	phlebotomus fever group	
Ar 1263	Anopheles	DCA resistant, ungrouped	
An 792	Arvicanthis	DCA resistant, related to Ar 662	
German Federal Republic			
ELB 4 and 5	man	patient had multiple sclerosis	
Iran			
Isfahan 91026-167	Phlebotomus	new member VSV group	new member VSV group
Iran 91039 (503095)	Argas	-	Abu Hammad
Iran 91064	Argas	ungrouped	
Iran 91005	Phlebotomus	Sicilian	
Iran 91045-M	Phlebotomus	Sicilian	
Iran 91045-AG	Phlebotomus	Karimabad	
Iran 91025-Q	Phlebotomus	Sicilian	
Iran 91025-C	Phlebotomus	Karamabad	
Japan			
Cap 15	Ornithodoros	ungrouped, same as Cap 44	
Cap 44	Ornithodoros	ungrouped	Nyamanini-related
Oita 293	bat	Rhabdovirus	new rhabdovirus

Table 44 cont.

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Korea			
RK	Rodent		
RAK 51	Rodent		
RAU 21	Rodent		
RAU 22	Rodent		
KK	Rodent		
Malaysia			
P72-4R	Monkey	ungrouped	new ungrouped virus
Panama			
Bradypus 121	Bradypus	Simbu group	Utinga-related, new
Bayano 000517	Culicoides	Simbu group	Utinga-related, new
Bradypus 4	Bradypus	ungrouped	ungrouped
322328	Haemagogus	yellow fever	
Rhodesia			
P25 (Mazoe)	Mastomys	ungrouped	viable
Senegal			
Ippy (Dak An D 188d)	Arvicanthis	ungrouped	
Cameroon-Mokola	Crocidura	Mokola	
South Africa			
Ar 13532	Culex rubinotus	Arumowot-related	
Ar 15908	Ornithodoros	ungrouped	Hughes group

Table 44 cont.

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Tanzania			
RML 64423-8	Argas	ungrouped	ungrouped
Uganda			
SG 37317/9	man	ungrouped	ungrouped
United States			
4 coded viruses		reidentification	LaCrosse, Snowshoe hare, Bunyamwera, Main Drain
VSV-New Jersey	prototype	reidentification	VSV-New Jersey
VSV-Indiana	prototype	reidentification	VSV-Indiana
VSV-Guatemala		reidentification	VSV-New Jersey
VSV-New Mexico	mosquitoes	reidentification	VSV-Indiana
Mass 2657-73		EEE	EEE
Mass Wilb 5627		EEE	EEE
Cal BFS 3112	Culex tarsalis	Oribivirus	
Cal BFN 3187	Culex tarsalis	Rhabdovirus	new rhabdovirus
Cal BRN 5662		Hart Park-Flanders	
RML 64423-8	Argas	ungrouped	ungrouped
1687 Oklahoma	monkey brain	Suspect rabies	not rabies

Table 44 cont.

Viruses referred to YARU for identification, 1976

Country of origin; strain	Source	Information from donor	YARU identification
Venezuela			
IVIC Pan 27014	sentinel hamster	EEE	
IVIC Pan 27011	sentinel hamster	EEE	
IVIC Pan 27029	sentinel hamster	EEE	
El Delirio	horse	EEE	
AROA	sentinel hamster	Group B	Group B

Table 45

Shipment of cell lines during 1976

Recipient	Cell line	Number of cultures	Month
Dr. Fred Ball, University of British Columbia, Vancouver, B.C., Canada	Aedes albopictus Aedes aegypti	2 4	March
Dr. Barney Cline, San Juan Laboratory, Puerto Rico	Aedes albopictus	3	March
Dr. P. Webb, CDC, Atlanta, Georgia	CER	2	March
Dr. Hazel Wallace, Hooper Foundation San Francisco, California	Aedes albopictus	3	April
Dr. Fabiyi, Virus Research Laboratory, University of Ibadan, Nigeria	Aedes albopictus Aedes aegypti	2 2	June
Dr. W. Neill, Gorgas Laboratory, Panama	CER	1	June
Dr. O. Wood, NAMRU-5, Field Facility, Addis Ababa, Ethiopia	Aedes albopictus	2	July
Dr. N. Karabatsos, Vector-borne Diseases Division, Center for Disease Control, Fort Collins, Colorado	CER	4	September
Dr. K. Mifune, Nagasaki University, Nagasaki, Japan	CER	1	December

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The Yale Arbovirus Research Unit serves the military and other agencies as an arbovirus reference center to identify viruses, serologically diagnose disease, prepare and distribute reference reagents and virus stocks, conduct serological surveys, and develop new techniques in arbovirology.

Virus and/or antiserum to 84 strains were received from 21 countries. From these Eyach virus from Germany was related to Colorado tick fever virus, new

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Kemerovo group viruses were identified from Denmark and Norway, a new phlebotomus fever group virus from the Central African Republic and several new tick-borne viruses from Japan, USSR, and Alaska.

Type specific California group sera were made in hamsters and sulfhydryl-agarose columns were applied to purification of mercury-tagged VEE RNA. Outbreaks of Korean hemorrhagic fever, chromosome-breakage in Yanomama Indians and Lyme (Connecticut) arthritis were investigated using serologic techniques. Arbovirus reference antigens and sera were supplied to requesting laboratories, including antigens to Walter Reed Army Institute of Research for study of fever cases from the Amazon region.

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